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STUDIES ON EMERGENCE AND SPREAD OF ANTIBIOTIC RESISTANT *STREPTOCOCCUS PNEUMONIAE*

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”Det är något bortom bergen, bortom blommorna och sången,
det är något bakom stjärnor, bakom heta hjärtat mitt.”

Dan Andersson

Till mitt underbara småfolk

ABSTRACT

Streptococcus pneumoniae is one of the major contributors to mortality and morbidity around the world. It causes a wide variety of diseases ranging from uncomplicated respiratory infections to life-threatening invasive infections such as meningitis and septicemia. In recent years, the effectiveness of antibiotic therapy has been hampered by the increasing rates of resistant pneumococci. As antibiotic resistance increases, there is a growing need for interventions that minimize opportunities for development and spread of resistant pneumococci. The aim of this thesis was to learn more about emergence and spread of antibiotic resistant pneumococci using both theoretical and empirical methods.

Since the increasing prevalence of resistant pneumococci is mainly due to the spread of strains belonging to few clones, interventions for controlling pneumococcal transmission in the community were studied. Model predictions suggested that interventions for efficiently control organism transmission should include reduction of group sizes in the day-care centers. Simulations also indicated that it appears extremely difficult to reduce the rates of penicillin non-susceptible pneumococci by simply decreasing the penicillin consumption assumed that reduced penicillin susceptibility does not imply a fitness cost for the organism. Managing the penicillin resistance rates in pneumococci then probably requires a more restrictive use of penicillin together with other control measures such as vaccine programs. Although clonal spread is the primary mechanism for the rapid emergence of resistance in pneumococci, natural competence for genetic transformation also seems to be involved. Further molecular understanding of competence regulation is important to be able to prevent horizontal spread of resistance genes. Studying the competence regulation by theoretical means led to the conclusion that down-regulation of competence is probably caused by a repressor acting on the *comCDE* operon at the level of transcription.

Despite the globally emerging frequency of resistant pneumococci, we conclude in a prospective study that antimicrobial resistance in invasive pneumococci in south-west Sweden remains limited. Any correlations between resistance pattern and clinical parameters could not be revealed. However, the serotype distribution was observed to differ between strains with reduced susceptibility and fully susceptible strains as isolates with decreased susceptibility more frequently belonged to the serotypes included in the 7-valent vaccine.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred in the text by their Roman numerals:

I. **D. Karlsson**, A. Jansson, B. Henriques Normark, and P. Nilsson. An individual-based network model to evaluate interventions for controlling pneumococcal transmission. BMC Infect Dis **2008**, 8:83.

II. **D. Karlsson.** Probabilistic network modelling of the impact of penicillin consumption on pneumococcal spreading. Submitted manuscript.

III. **D. Karlsson**, S. Karlsson, E. Gustafsson, B. Henriques Normark, and P. Nilsson. Modeling the regulation of the competence-evoking quorum sensing network in *Streptococcus pneumoniae*. BioSystems **2007**, 90(1):211-23.

IV. E. Backhaus, S. Berg, B. Trollfors, R. Andersson, E. Persson, B. E. Claesson, P. Larsson, E. Ek, L. Jonsson, G. Rådberg, S. Johansson, T. Ripa, **D. Karlsson**, and K. Andersson. Antimicrobial susceptibility of invasive pneumococcal isolates from a region in south-west Sweden 1998-2001. Scand J Infect Dis **2007**, 39(1):19-27.

CONTENTS

LIST OF ABBREVIATIONS

AOM	Acute otitis media
CAP	Community-acquired pneumonia
CPS	Capsular polysaccharide
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CSP	Competence-stimulating peptide
DCC	Day-care center
I	Indeterminate resistant
Ig	Immunoglobulin
IPD	Invasive pneumococcal disease
LTA	Lipoteichoic acid
MHC	Major histocompatibility complex
MIC	Minimum inhibitory concentration
MLS_B	Macrolide, lincosamide, and streptogramin B
ODE	Ordinary differential equation
PBP	Penicillin-binding protein
PCV	Pneumococcal conjugate vaccine
PG	Peptidoglykan
PNSP	Penicillin non-susceptible pneumococci
R	Resistant
S	Sensitive
TA	Teichoic acid
TLR	Toll-like receptor
TMP-SMX	Trimethoprim-sulphametoxazole
WHO	World Health Organization

1 INTRODUCTION

1.1 GENERAL MICROBIOLOGY OF *STREPTOCOCCUS PNEUMONIAE*

The pneumococcus (*Streptococcus pneumoniae* or diplococcus) was first isolated independently by Louis Pasteur and George Miller Sternberg in 1880. Ever since that time, research on this organism has resulted in advancements in our understanding of the human response to infectious diseases, natural transformation as a mean of genetic exchange, bacterial resistance to antibiotics, as well as vaccine-related immunoprophylaxis. In past decades, research on pneumococci has even been intensified due to the pneumococcal acquirement of multiple antibiotic resistances, and to the development of novel vaccines to counter this problem. Nevertheless, pneumococcal diseases remain a leading cause of morbidity and mortality around the world, and still there is much to be discovered about the biology of this organism.

1.1.1 Pneumococcal physiology and structure

S. pneumoniae is a Gram-positive, facultative anaerobe that grows as single cells, diplococci or in short chains. The organism is an oval or spherical coccus of 0.5-1.25 μm in diameter. The pneumococci are α-hemolytic as signified by their formation of greenish haloes on blood agar plates. The α-hemolysis, also called partial or incomplete hemolysis, is caused by the hydrogen peroxide produced by the bacteria. The hydrogen peroxide oxidizes hemoglobin to green methemglobin. The pneumococcal genome corresponds to a circular chromosome containing about two million base pairs depending on the strain. Around 2,000 genes are encoded by the pneumococcal chromosome.

The pneumococci are surrounded by a thick polysaccharide capsule which covers the cell wall. The capsular polysaccharides (CPS) represent a diverse group of polymers that play an important role in the virulence of the organism. The polysaccharides differ in both their sugar compositions and linkages. Despite this diversity, all capsular types perform the same primary function of protecting against phagocytic clearance by blocking the deposition and function of opsonins directed against cell surface antigens (Abeyta *et al.* 2003; Hostetter 1986; Szu *et al.* 1983). Thus, non-encapsulated strains have less protection and thereby also greatly reduced virulence (Griffith 1928). The antigenicity of the capsule forms the basis for the classification of different capsular serotypes. Hitherto, 48 different serogroups have

been described, which are further subdivided into a total of 91 serotypes (Park *et al.* 2007). The serotypes are distinguished by the ability of the immune system in rabbits to recognize chemical differences in the CPS and to respond with specific antibodies against antigens of each different type. The system of nomenclature, generally used for the pneumococcal serotypes, is referred to as the Danish system and is based on cross-reactions between different serotypes (Kamerling 2000). Serologically cross-reactive serotypes are assigned to a common serogroup with individual serotypes within each group distinguished by a trailing letter. The number assignment refers to the serogroup, e.g., serogroup 7, which is subdivided into the serotypes 7A, 7B, etc. The host's protective antibodies are directed against the polysaccharide capsule, and the immune response is therefore type/group specific with only slight or no cross-reactivity between antibodies of different specificity. The role of the pneumococcal polysaccharide capsule as virulence factor is further discussed in section 1.5.1.

1.1.2 Competence for natural transformation

Transformation, which alters the genetic makeup of an individual, was first discovered in *S. pneumoniae* in 1928 by Frederick Griffith. At that time, numerous serologic types had been recognized in pneumococci. In Griffith's laboratory, the research was focused on the variation in capsule involved in the serologic differences. He reported that heat-killed encapsulated pneumococci could transfer the ability to produce a capsule and to infect mice when injected together with live, unencapsulated (non-pathogenic) pneumococci (Griffith 1928).

Competence for natural genetic transformation of the type found in pneumococci has been reported in about 70 different species of bacteria (Johnsborg *et al*. 2007), for example *Haemophilus influenza*, *Bacillus subtilis, Neisseria meningitidis*, and *Neisseria gonorrhoeae*. Natural transformation requires a set of genes evolved for the purpose, in contrast to artificial transformation, which is accomplished by shocking cells either electrically or by ionic strength and temperature shifts. Such treatments can introduce very small amounts of DNA into any type of cell. The genetic material introduced by natural transformation is a million fold greater; *S. pneumoniae* can take up as much as 10% of its cellular DNA content (Hotchkiss 1954).

The regulation of competence differs between bacterial species. In *N. gonorrhoeae* the competence is a constitutive feature, whereas in *H. influenzae* it is induced by starvation (Solomon *et al.* 1996). In both *S. pneumoniae* and *B. subtilis* the development of competence is regulated by cell-cell signaling. More precisely, the

initiation of competence in pneumococci is regulated by a peptide-controlled quorum sensing system called ComABCDE pathway (Figure 1).

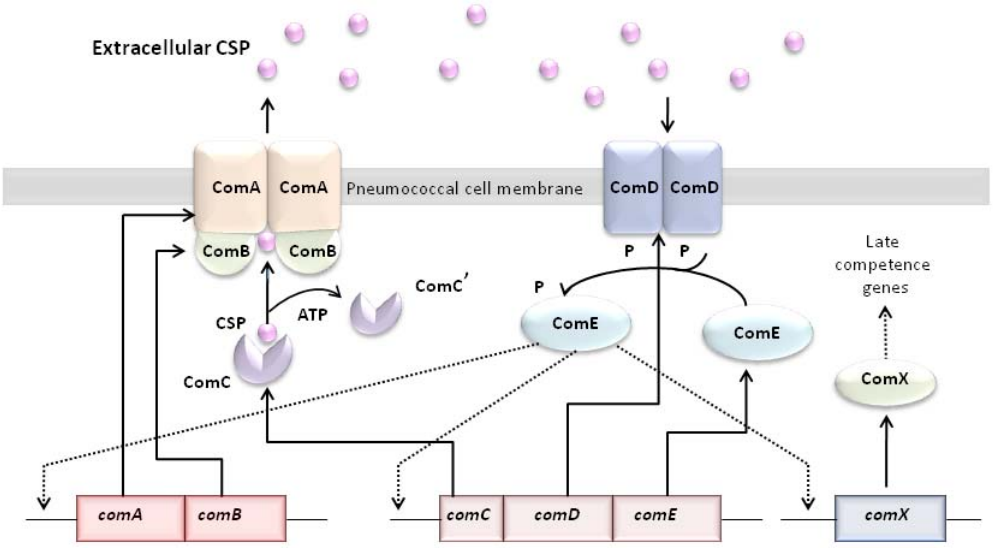

FIGURE 1 Model of the quorum sensing regulated competence pathway in *S. pneumoniae.* The signal peptide CSP accumulates extracellularly and binds to its receptor, ComD. Binding of CSP elicits a conformational change in ComD leading to autophosphorylation. ComD donors the phosphate group to the response regulator ComE which then increases its efficiency as transcription factor significantly. The alternative sigma factor ComX is the unique link between quorum sensing and competence development.

The quorum sensing signal is a heptadecapeptide called competence-stimulating peptide (CSP), which is encoded by the 3´ moiety of *comC* (Havarstein *et al.* 1995). The signal peptide is exported by an ABC-transporter, ComAB, which recognizes and cleaves the amino-terminus of CSP (Hui *et al.* 1995). CSP then accumulates extracellularly and is sensed by a membrane-bound histidine kinase, ComD, which autophosphorylates upon binding CSP. Thereafter, ComD transfers the phosphate group to its response regulator, ComE, which functions as a transcriptional activator (Ween *et al.* 1999). Phosphorylation of ComE enhances the affinity for its binding site, a conserved direct-repeat motif in the promoter regions of the target genes, leading to an increased expression of about 20 early competence genes. Binding sites for ComE have been identified upstream of both *comAB* and *comCDE* operons (Ween *et al.* 1999), and for *comX*, encoding an alternative sigma factor that has been reported as a unique link between quorum sensing and competence development (Luo *et al.* 2003a; Luo *et al.* 2003b). The expression of late *com* genes, which encodes proteins involved in binding, uptake, processing, and recombination of exogenous DNA, is dependent on

ComX which acts by replacing SigA in RNA polymerase (Campbell *et al.* 1998; Martin *et al.* 1995; Pestova *et al.* 1998; Peterson *et al.* 2004).

Pneumococcal cells are not always competent to take up DNA. In batch cultures, competence is induced when CSP reaches an external concentration of 1-10 ng ml^{-1} (Havarstein *et al.* 1995) corresponding to a cell density between 10^5 and 5 x 10^8 cell ml^{-1} (Chen *et al.* 1987). Different factors of environment and metabolism such as temperature, pH (Chen *et al*., 1987), cations (Fox *et al*. 1957), and oxygen concentration (Echenique *et al*. 2000) explain the wide range in cell density at which competence can be initiated in a batch culture. The majority of non-competent cells in an exponential phase are affected by CSP-induced competence (Havarstein *et al.* 1995). In experimental studies, waves of competence have been observed before the culture reaches the stationary phase. However, in the second wave around 12% of the cells become competent and any additional waves only involve a minority of the cells (Chen *et al.* 1987; Morrison 1997). Studies on the laboratory strain Rx have demonstrated that competence appears as a sharp peak of around 30 min in duration. To date, the mechanisms responsible for the shut-off of competence shortly after its induction is not clarified and further research is required. However, we examined theoretically the competence system regarding plausible mechanisms involved in the competence shut off (**paper III**). Model results suggested that down-regulation of competence probably is caused by a hypothetical repressor acting on the *comCDE* operon at level of transcription.

For transformation to occur under natural conditions, DNA must be released from donor cells as well as being taken up by recipient cells. Probably, the transforming DNA originates from lysed non-competent pneumococcal cells where lysis was triggered by competent cells (Guiral *et al.* 2005). During the competence development, pneumococci express a putative murein hydrolase, CbpD, which mediates lysis of non-competent target cells in the surrounding environment (Eldholm et al. 2009). The competent attacker cells, which protect themselves against their own lysins by an immunity protein encoded by the *comM* gene, are able to take up the DNA released from the lysed cells (Håvarstein et al. 2006). The exact mode of actions of CbpD and ComM has not been determined yet. The major pneumococcal autolysin, LytA, appears to be constitutively expressed in pneumococci, but during competence development its expression is enhanced (Peterson *et al*. 2004). Another cell wall hydrolase, LytC, has also been reported to contribute to the lysis mechanism although it is not a part of the competence regulon (Eldholm *et al*. 2010).

The possible benefits of natural competence in bacteria have been discussed, but competence is probably advantageous because of its role in horizontal gene transfer and it might also aid in the repair of damaged chromosomes (Solomon *et al.* 1996). Since natural transformation in *S. pneumoniae* mediates intraspecies as well as interspecies gene transfer, it gives the pneumococcus access to a large gene pool, which it shares with other pneumococcal strains and closely related streptococci (Hiller *et al*. 2007). Under certain types of stress such as antibiotics, genes present in the common gene pool that give a selective advantage will spread rapidly among these bacteria. In pneumococci, natural transformation is most likely to be responsible for serotype-switching, and has also influenced the evolution of virulence factors (Coffey *et al.* 1991; Coffey *et al.* 1998; Hollingshead *et al.* 2000; Kelly *et al.* 1994; Ween *et al.* 1999) and the rapid emergence of antibiotic resistance (Coffey *et al.* 1991; Dowson *et al.* 1989; Hakenbeck *et al.* 1998).

1.2 PNEUMOCOCCAL CARRIAGE AND TRANSMISSION

The human nasopharynx is the primary reservoir for *S. pneumoniae* and the main source of person-to-person transmission. Pneumococcal carriage is mostly transient and asymptomatic, but constitutes as well a prerequisite to pneumococcal disease. In contrast to diseases that do not have a carrier state, asymptomatic carriers of pneumococci contribute more to pneumococcal transmission than infected individuals. Transmission occurs from person-to-person via droplets meaning that close contact is required for transmission to occur.

Because the preconditions for acquiring resistance by transformation and conjugative transposons are stringent and *de novo* resistance for the majority of antibiotic classes does not result from a single point mutation, the evolution of novel resistant pneumococci from an initially sensitive strain occurs only rarely. However, once in existence and under the selective pressure of antibiotics, resistant strains are able to spread widely. For most of the major antibiotic classes, resistant pneumococci are spread primarily by clonal amplification. Consequently, infections caused by resistant pneumococci result mostly from the acquisition of resistant strains from other nasopharyngeal carriers in the surrounding.

Primarily children are colonized by *S. pneumoniae*, whereas adults seldom are carriers. In Sweden, between 15-50% of the children are colonized, depending on age

and frequency of contacts with other children (Borres *et al.* 2000; Söderström 1990). Among the adults, around 3% habit pneumococci in nasopharynx (Kalin 1982). In developing countries, pneumococcal carriage is even more common; as many as 85% of the young children can be colonized (Huebner *et al.* 1998). Seasonal variations in nasopharyngeal carriage have been observed; it is more common to be colonized in the winter months compared with summer season (Hendley *et al.* 2005; Marchisio *et al.* 2001).

The duration of carriage varies depending on the host's age and on the serotype of the colonizing strain (Ekdahl *et al.* 1997; Hogberg *et al.* 2007). Typically, the carriage duration ranges from one month up to a year. It appears to be an inverse relationship between age and duration (Ekdahl *et al.* 1997; Hogberg *et al.* 2007). Isolates belonging to serotype 19F, 23F, 6B, and 35F have been reported to be carried in the nasopharynx for 13-32 weeks, whereas serotype 12F, 3, 15B/C, and 9V reside for five to eight weeks (Sleeman *et al.* 2006). Duration of carriage for serotype 1, 4, 5, and 9A could not be determined since the sampling intervals were longer than two weeks. These serotypes obviously inhabit the nasopharynx for a shorter time (Sleeman *et al.* 2006). In concurrence, other studies have reported that serotype 1 and 4 are rare or absent among carriers, whereas serotype 6A, 6B, 19F, and 23F are more commonly found (Brueggemann *et al.* 2003; Henriques Normark *et al.* 2003; Sandgren *et al.* 2004).

Nasopharyngeal carriage varies by geographic region. Perhaps the variation in the prevalence of nasopharyngeal carriage is due to genetic differences in the host that influence the likelihood of nasopharyngeal colonization and also to socioeconomic conditions such as crowding, hygiene, family size, and day-care contact (Schrag *et al.* 2000).

1.3 CLINICAL DISEASES

The spectrum of diseases caused by *S. pneumoniae* is wide, from mild, mucosal infections such as sinusitis and otitis media to more severe, invasive diseases as septicemia and meningitis. The factors involved in the transition from carriage to disease remain inadequately understood. Existing data indicate that the risk for disease progression is greatest soon after exposure and acquisition of pneumococci in nasopharynx (Gray *et al*. 1980). The risk for disease progression differs between ages and different disease manifestations. Currently, pneumococcus is the most common

etiological agent causing otitis, sinusitis, and community-acquired pneumonia (CAP) (Iwarson-Norrby 2007). The majority of pneumococcal diseases occur when organisms are spread from an area that is colonized into a space that is normally not colonized, such as Eustachian tube, paranasal sinus, bronchiole, alveolus, or fallopian tube. The most common pneumococcal diseases are described more in detail in this section. In addition to these clinical manifestations, pneumococci may also cause other infections, although more rarely, such as conjunctivitis, septic arthritis, and endocarditis, etc.

1.3.1 Sinusitis

Sinusitis is a group of disorders where the sinuses and nasal passages are inflamed. The most common direct cause of acute sinusitis is bacteria. In children and elderly, acute bacterial sinusitis is an uncommon disease; around 90% of the cases are observed in the ages between 15-65 years (Iwarson-Norrby 2007). About half of the sinusitis cases are caused by pneumococci; other important pathogens include *H. influenzae* and *Moraxella catarrhalis*. The symptoms for acute sinusitis may vary but is often manifested by purulent nasal drainage, nasal obstruction, fever, cough, headache, and facial pain-pressure. The location of pain depends on which sinus is affected.

1.3.2 Otitis media

Acute otitis media (AOM) is an infection in the middle ear, often caused by bacteria. It is one of the most frequent diagnoses in young children in Sweden; at the age of four years, around half of the children have experienced at least one otitis and nearly a quarter have had two or more episodes (Iwarson-Norrby 2007). The frequency declines with increasing age and is rather uncommon after the age of six years. The bacteria most often involved in otitis belong to the commensal flora of the naso- and oropharynx. In Sweden, the dominating etiologic agent for otitis is *S. pneumoniae*, causing between 30-50% of the cases, followed by *H. influenzae*, *M. catarrhalis,* and sometimes *Streptococcus pyogenes* (Iwarson-Norrby 2007).

Otitis media is defined as the presence of effusion behind the tympanic membrane within the middle ears, whereas AOM is an infection characterized by rapid onset of symptoms of middle ear inflammation and effusion. Common symptoms are fever, otalgia, headache, hearing impairment, nausea, balance problems, purulent effusion in the middle ear, and inflammation or bulging of the tympanic membrane (Lieberthal 2006; Ramakrishnan *et al.* 2007). Otitis media has a very low mortality, although there are a numbers of more severe complications such as meningitis, acute

mastoiditis, reduction in hearing or total hearing loss, and chronic otitis media, which may affect children. Persistent morbidity due to otitis media is more common in developing countries due to lack of antibiotics (Klein 2000). Even a successful treatment of AOM, i.e., eradication of isolates from the middle ear fluid, may result in recurrent AOM caused by nasopharyngeal carriage since antibiotic does not always clear the nasopharynx from pneumococci (Libson *et al.* 2005).

1.3.3 Pneumonia

Pneumonia is defined as an inflammation, often infection-triggered, of one or both lungs with consolidation. The most common etiological agent of CAP is the pneumococcus, which is believed to be responsible for 25-45% of the cases which in turn corresponds to 60-70% of all bacterial CAP (Iwarson-Norrby 2007; Lutfiyya *et al.* 2006). Examples of other common bacterial causes of CAP include *Mycoplasma pneumoniae*, *H. influenzae, Legionella* spp., and *Chlamydia pneumoniae* (Iwarson-Norrby 2007). Pneumococcal pneumonia can affect all age groups. The clinical signs of pneumonia vary depending on age and causative agent as well as other factors. However, the most frequent symptoms of pneumonia among elderly children and adults are cough with sputum production, fatigue, fever, chills, sweats, and shortness of breath (Buttery *et al.* 2002). In very young children and the elderly, the symptoms are often non-specific which make it difficult to diagnose the disease (Andrews *et al.* 2003).

In contrast to CAP, nosocomial pneumonia is aquired at hospitals or long-term care facilities. It can be caused by various agents, for example *Staphylococcus aureus, Pseudomonas aeruginosa* and Gram-negative enterobacteria, which of the relative frequencies vary with geographic locations, seasons, age of the patient, etc (Iwarson-Norrby 2007). The pneumococcus may also cause nosocomial pneumonia, especially among elderly people in long-term care facilities (Iwarson-Norrby 2007).

1.3.4 Septicemia

Septicemia, also referred to as sepsis, is a serious medical condition characterized by a whole-body inflammatory state, termed a systemic inflammatory response syndrome, and the presence of an infection. Systemic inflammatory response syndrome can be indicated by symptoms like fever >38^{o}C or < 36^{o}C, respiratory rate >20 breaths/min, pulse more than 90 beats/min, and white blood cell count <4,000 cells/mm^3 or >12,000 cells/mm^3. For a sepsis characterization, at least two of these symptoms most be presented (Nguyen *et al.* 2006). There are different levels of sepsis and a classification

system has been established, which includes sepsis, severe sepsis, and septic shock. The symptoms of bacterial sepsis are mainly due to an immunologic hyper reaction occurring when bacteria enter the blood stream and thereby activate macrophages and other cells to release cytokines, such as interleukin-1 and tumor necrosis factor. In massive infections, the production of cytokines reaches uncontrollable and detrimentally high levels systematically. The high levels of these proinflammatory proteins induce the systemic effects of sepsis. This response triggers the complement system and coagulation pathways which then cause damage to the vasculature as well as to the organs. Typical clinical symptoms consist of high fever or hypothermia, hypotension, shivering, tachycardia, skin rashes, and tachypnoea. In the case of septic shock, the symptoms also include increased vasopermeability and vasodilation, which may progress to organ failure and death.

The dominating infectious agent for bacterial sepsis depends on the age. During the first week after birth, infections with *Escherichia coli*, group B streptococci and *Listeria monocytes* are dominating, whereas during the childhood and up to 20 years, pneumococci, *N. meningitidis*, and *S. aureus* are more common (Iwarson-Norrby 2007). In adult patients, septic infections are most often caused by Gram-negative enterobacteria (Iwarson-Norrby 2007). The pneumococcus is estimated to be the causative agent in around 10% of the cases of severe sepsis and septic shock (Iwarson-Norrby 2007).

1.3.5 Meningitis

Meningitis is an inflammation of the meninges surrounding the brain and spinal cord. It can be caused by viruses, bacteria or other microorganisms. Which types of bacteria that commonly cause bacterial meningitis vary between age groups. Pneumococcus is a common etiological agent for meningitis occurring in very young children between one month to one year of age and in adults older than 40 years (Iwarson-Norrby 2007). Examples of other causative agents include *N. meningitidis*, *E. coli*, group B streptococci, *L. monocytes,* and *H. influenzae* (van de Beek *et al.* 2004). Bacterial meningitis often arises secondarily to an infection focus outside the central nervous system; pneumococcal meningitis can for example be secondary to AOM (so called otogenic meningitis) or to respiratory tract infection (Iwarson-Norrby 2007). Typically, symptoms come quickly and may include high fever, severe headache, stiff neck, confusion, nausea, photophobia, and general signs of illness. The mortality rate for pneumococcal meningitis is high, ranging from 15% to 60% (Burman *et al.* 1985; Hoen

et al. 1993; Kornelisse *et al.* 1995; Kragsbjerg *et al.* 1994; Traore *et al.* 2009). Besides, the survivors of pneumococcal meningitis are more likely to suffer from sequele, such as hearing loss, neurological complications or neurophysiological impairment, than survivors of other forms of meningitis (Jit 2010)

1.4 PATHOGENESIS

The pathogenesis of pneumococcal infection is a complex interplay between pneumococcal virulence factors and the host immune response. Even if *S. pneumoniae* possess a collection of characteristics that allow it to colonize a variety of niches, the disease manifestations are caused mainly by the host response rather than the production of toxic factors. Principally, all pneumococcal infections begin with colonization of the nasopharynx. Several interacting factors determine whether the colonizing organisms go on to establish infection of the lower respiratory tract. Following the colonization of nasopharynx, the bacteria may spread to the lungs, the paranasal sinuses, or middle ear (Figure 2). It can also be transported in the blood to the brain.

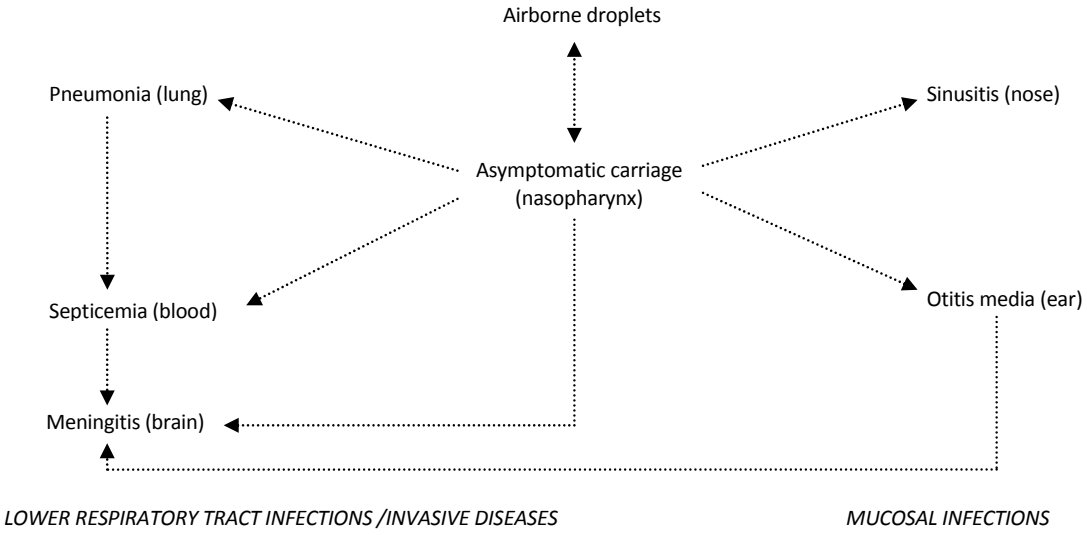

FIGURE 2 Pathogenic routes for pneumococcal infection. A prerequisite for pneumococcal disease is colonization of nasopharynx. From here, the bacteria can spread and cause mucosal infections such as otitis media and sinusitis, but also more serious conditions like pneumonia, septicemia, and meningitis can arise.

Initially, the colonization of nasopharynx is mediated by the attachment of the bacteria to epithelial cells by means of surface protein adhesins. Pneumococcal factors that have

been indicated to promote binding to epithelial cells include choline, pneumococcal neuraminidase, and CbpA (Cundell *et al.* 1995a; Rosenow *et al.* 1997; Tong *et al.* 2000). On the host epithelial cells, the target molecules are usually sugars as disaccharides and sialic acid, but the platelet-activating factor receptor can be used as well (Cundell *et al.* 1995a). If the organisms are enveloped in mucus and removed from the airways by the action of ciliated epithelial cells, subsequent migration of the pneumococci to the lower respiratory tract can be prevented. However, this mucus envelopment is counteracted by the bacteria by their production of immunoglobulin (Ig) A protease and pneumolysin (Kornfeld *et al*. 1981). Human secretory IgA acts by trapping bacteria in mucin by binding itself to the bacteria at the antigen-binding site and to mucin. This interaction can be interfered by bacterial IgA proteases which are capable of cleaving secretory IgA. Pneumolysin also plays an important role in evading the host defense by binding to cholesterol in the host cell membrane and causing pores, thereby destroying ciliated epithelial cells and phagocytic cells.

Pneumococcal colonization has been reported to correlate with observed differences in colony phenotypes. This human pathogen seems to undergo spontaneous, reversible opacity phase variation with a frequency of 10^{-3} to 10^{-6} resulting in opaque and transparent colonies (Weiser *et al.* 1994), where the transparent phenotype produces less CPS and has an enhanced ability to reside on mucosal surfaces when tested in animal models (Tong *et al.* 2001). By contrast, it appears that the opaque variant is more virulent in systemic infections probably because the higher levels of CPS production make them more resistant to complement-mediated opsonophagocytotic elimination (Kim *et al.* 1998).

Presumably, pneumococci spread to the lungs by aspiration. It has been indicated that the attachment of pneumococci occurs to bronchial epithelial cells (Adamou *et al.* 1998) or to components of the basement membrane including laminin, collagen and fibronectin (Kostrzynska *et al.* 1992; van der Flier *et al.* 1995). An impaired ciliary beating frequency due to damage caused by the release of pneumolysin or by smoking, promotes the binding of pneumococci. Pneumolysin inhibits the normal beating of cilia by destroying epithelial and endothelial cells and thereby making it easier for the organisms to enter the blood stream (Steinfort *et al.* 1989; Zysk *et al.* 2001). Following influenza virus infection, adherence of pneumococci to tracheal epithelial cells is enhanced by viral neuraminidase (Plotkowski *et al.* 1986). This enzyme cleaves sialic acid from glycosphingolipids in human lung tissue and thereby exposing other structures that function as receptors for adhering pneumococci (Krivan

et al. 1988). Viral infections may also facilitate the binding of pneumococci by affecting epithelial cells to upregulate the platelet-activating factor receptor and by damaging bronchial epithelium (McCullers *et al.* 2010; Obaro *et al.* 1996).

Inside the alveoli, pneumococci bind to type II pneumocytes and can spread rapidly into the blood by crossing the vascular endothelium (Tuomanen *et al.* 1995). Once the bacteria have reached blood stream, several factors are required. The most essential is the antiphagocytotic capsule, but pneumolysin, pneumococcal surface protein A, and CbpA also play an important role by the interfering with complement activation (Cheng *et al.* 2000; Mitchell *et al.* 1991; Tu *et al.* 1999). During pneumococcal pneumonia, three different stages of lesions were distinguished: (1) engorgement, (2) red hepatisation, and (3) gray hepatisation. The first stage, engorgement, is associated with the accumulation of a serous exudate in the alveoli. Thereafter, a leakage of erythrocytes into the alveoli occurs. During the last stage, bacterial multiplication peaks and fibrin is formed through the procoagulant activity induced by pneumococci. Furthermore, neutrophils recruited to the infection site begin to control pneumococcal multiplication (Tuomanen *et al.* 1995). However, clearance of pneumococci by opsonophagocytosis depends further on complement and is facilitated by anti-capsular antibodies (AlonsoDeVelasco *et al.* 1995). The tissue destruction often observed in pneumococcal pneumonia is primarily due to the host´s inflammation response, thereof the remarkable feature of pneumococcal pneumonia that the lungs of surviving patients almost invariably return to normal, irrespective of the severity of the systemic or pulmonary condition when the disease was at its peak (Catterall 1999).

1.5 VIRULENCE FACTORS

Over the years, several pneumococcal virulence factors have been identified, some more investigated than other. In this section, some of the major and most well-known virulence factors are to be described (Figure 3).

1.5.1 Polysaccharide capsule

The capsule has been recognized as the principal virulence factor of *S. pneumoniae*. Encapsulated strains have been found to be at least 10^5 times more virulent than strains lacking capsule (Watson *et al.* 1990). Basically all clinical isolates possess a capsule, and non-encapsulated mutants are considered avirulent (Griffith 1928; Watson *et al*. 1990). Based on the chemical structure of the capsules more than 90 different capsular

types have been described so far (Park *et al.* 2007). The capsule consists of a mix of repeating units of polysaccharides and varies in complexity. The main role of the capsule is to protect against phagocytosis by macrophages and neutrophils by inhibiting C3b opsonisation of the pneumococcus. The chemical structure and, to a lesser extent, the thickness of the capsule seems to be important determinants for the differential ability of serotypes to survive in the bloodstream and possibly to cause IPD. Studies have also shown that the capsule is essential for colonization (Magee *et al.* 2001; Nelson *et al.* 2007).

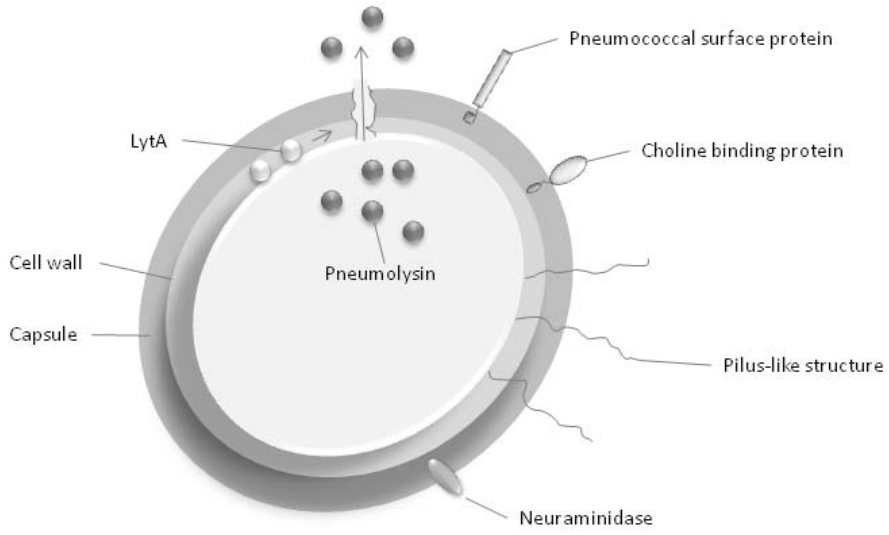

FIGURE 3 Some of the major pneumococcal virulence factors including polysaccharide capsule, cell wall fragments (PG, TA, and LTA), LytA, pneumolysin, pneumococcal surface proteins, choline binding proteins, and neuraminidase. Pilus-like structures are found in some pneumococci.

1.5.2 Cell wall fragments

The pneumococcal cell is also surrounded by a cell wall, which is a thick multi-layered structure built up mainly by peptidoglycan (PG), teichoic acid (TA), and lipoteichoic acid (LTA). Studies have shown that pneumococcal cell wall preparations induce inflammation similar to what has been observed with whole pneumococci (Fischer *et al.* 1993; Mosser *et al.* 1970). Otitis media, meningitis, and pneumonia are examples of typical pneumococcal diseases that have been demonstrated to be mimicked in animals, which have received injection of purified cell wall or its degradation products (Carlsen *et al.* 1992; Tuomanen *et al.* 1985; Tuomanen *et al.* 1987). Cell wall fragments activate the alternative pathway of complement (Winkelstein *et al.* 1977; Winkelstein *et al.*

1978). The anaphylatoxins C3a and C5a, which enhance vascular permeability, provoke mast cell degranulation, recruit and activate polymorphonuclear leukocytes at the site of inflammation, are produced during complement activation (Johnston 1991). Several studies have reported that anti-cell wall polysaccharides or anti-phosphorylcoline antibodies protect animals against pneumococcal challenge (Briles *et al.* 1981; Briles *et al.* 1992; Briles *et al.* 1989; Kenny *et al.* 1994; Nicoletti *et al.* 1993), whereas such a protection was not observed by other (Nielsen *et al.* 1993; Szu *et al.* 1986).

1.5.3 Pneumolysin

Pneumolysin is an intracellular thiol-activated toxin present in almost all clinical isolates that has a variety of toxic effects on different cell types. Previously, pneumolysin was thought to not be secreted by pneumococci, only released upon lysis of the bacterium under the influence of autolysins. However, in 2001, a secretion mechanism for pneumolysin was suggested (Balachandran *et al.* 2001). This toxin is believed to have multiple functions in virulence as it possesses both cytotoxic and pro-inflammatory properties. At high concentration, pneumolysin binds to cholesterol in host cell membranes and may disrupt them by forming transmembrane pores. At lower concentrations, it stimulates the production of inflammatory cytokines such as tumor necrosis factor-α and interleukin-1β (Houldsworth *et al.* 1994), decreases the bactericidal activity and migration neutrophils (Paton *et al.* 1983), and activates the classical complement pathway by binding to the Fc portion of nonspecific antibodies and thereby causing inflammation tissue damage (Mitchell *et al.* 1991).

1.5.4 Autolysins

Autolysins, also called bacterial cell wall hydrolases, are enzymes that specifically cleave bonds of the cell wall. This cleavage makes the cell unstable and sensitive to osmotic pressure that causes lysis and death of the bacteria. The major autolysin in pneumococci is LytA, a choline-binding protein located in the cell wall. LytA is required for daughter cell separation and pneumococcal lysis in stationary phase as well as in the presence of penicillin. In 1989, it was shown that LytA-negative mutants were less virulent than wild-type pneumococci (Berry *et al.* 1989). However, the effect of LytA seems to be mediated by the release of pneumolysin from the pneumococcal cytoplasm during infection because immunization with autolysin does not protect against challenge with a pneumolysin-negative mutant (Lock *et al.* 1992). Analyses of

expression data have shown that the *lytA* gene is over-expressed during competence development (Peterson *et al*. 2000). The reason for this finding is still unclear as it is established that LytA is not required for transformation (Sánchez-Puelles *et al*. 1986). Two other autolysins, LytB and LytC, are also described but their functions are less understood.

1.5.5 Phase variation

Weiser *et al*. showed in 1994 that *S. pneumoniae* is able to make two different colony types, a transparent colony type and an opaque colony type when grown on agar plates (Weiser *et al.* 1994). The impact of this reversible phase variation has been further examined since then. It has been suggested that the pneumococcus phase varies between a virulent opaque form expressing more CPS and less TA, and an avirulent transparent form with less CPS and more TA (Kim *et al.* 1998). Probably, the different appearances in colonies reflect differences in surface molecules that may mediate adherence. Strains with the transparent colony type adhere much more tightly to human epithelial cells *in vitro* and are also more potent colonizers in animal models (Cundell *et al.* 1995b; Weiser *et al.* 1994). More precisely, the enhanced colonization of the nasopharynx *in vivo* by transparent phase variants involves a greater ability to adhere to GlcNAc (*N*-acetylglucosamine) which is a major receptor during colonization (Cundell *et al.* 1995b). In systemic infections, the virulent opaque variant has been found to be more resistant against complement-mediated opsonophagocytotic elimination by the high-level expression of CPS (Kim *et al.* 1998).

1.5.6 Pilus-like structures

Recently, pilus-like structures were recognized in pneumococci (Barocchi *et al.* 2006). These pili were found to be encoded by the *rlrA* pathogenicity islet and appear to be present in some, but not all clinical isolates. A study from 2008, reported that only a minority, around 27%, of clinical invasive isolates harbored the pilus islet which indicates that it is not an essential virulence factor (Aguiar *et al.* 2008). However, piliated pneumococci have been demonstrated to be more virulent as piliated pneumococci have been shown to outcompete non-piliated mutants in a mouse model of colonization, pneumonia, and bacteremia (Barocchi *et al.* 2006).

1.6 HOST RESPONSE

1.6.1 Immune system

Most symptoms of pneumococcal disease are generated by the host inflammatory response. The host immune response is composed of two major subdivisions, the innate immunity and the adaptive immunity. The innate immune system provides a rapid, non-specific first line of defense against invading microbes. It includes immune cells such as macrophages, neutrophils, and natural killer cells as well as specialized chemical mediators as cytokines, and the complement cascade. It is present from birth and does not require pre-exposure to foreign microbial material to react. Innate immunity can directly react against a large number of microbes by recognizing highly conserved molecular microbe specific motifs such as bacterial PG and LTA. The innate recognition is achieved through a limited set of germline-encoded receptors and does not possess immunological memory. These highly conserved molecular motifs are also called pathogen-associated molecular patterns and are recognized by Toll-like receptors (TLRs) and other pattern recognition receptors such as the soluble acute phase protein called lipopolysaccharide-binding protein (Weber *et al.* 2003) and the cytosolic NOD-like receptors. Many TLRs have been implicated to be involved in the recognition of pneumococci. For example, TLR4 has been shown to recognize pneumolysin (Malley *et al.* 2003) whereas TLR2 seems to recognize pneumococcal LTA as well as PG (Koedel *et al.* 2003). Another well-known example of pattern recognition receptors involved in the response to the pneumococcus is C-reactive protein (CRP). This soluble protein binds phosphorylcholine located in the cell wall and thereby induces complement activation and bacterial destruction

A part of the innate immunity, which plays an important role in clearance of the bacteria during a pneumococcal infection, is the complement system. It consists of more than 30 serum and membrane proteins which, when activated, form an enzymatic cascade of reactions contributing to the elimination of invading microbes. The binding and activation of complement proteins to the bacterial surface results in opsonophagocytosis and inflammation induction. Opsonophagocytosis appears to be the major immune mechanism protecting the host against pneumococcal infection (Bruyn *et al.* 1992). Complement can also destroy some microorganisms, however, not the pneumococcus, directly through lysis by the membrane attack complex. Humans with deficiencies in the complement pathways have been reported to have an increased susceptibility to pneumococcal infections (Picard *et al.* 2003; Revest *et al.* 2009; Totan

2002). Three different activation pathways of the complement have been identified. However, the classical pathway of activation is the dominating one for pneumococci, where complement is activated by antigen-antibody complexes or the binding of acute-phase proteins, for example CRP, to the bacterium (Brown *et al.* 2002). To a lesser degree than the classical pathway, the alternative pathway also appears to contribute to innate protection, whereas the role for the lectin pathway seems negligible. The deposition and activation of the complement component C3 on the surface of the microbe is a key step in the enzymatic cascade that will clear the bacteria. In accordance with the importance of this component, the pneumococcus has evolved mechanisms to resist its affect. A crucial determinant in this resistance is the capsule which acts to limit the access to cell bound complement and to reduce the amount of complement deposited as well (Abeyta *et al.* 2003).

The innate immune system also constitutes the link to the adaptive immune system consisting of antigen-specific reactions through T-cells and B-cells. The adaptive immunity possesses a memory so that subsequent exposure leads to a stronger and faster response. When extracellular pathogens such as pneumococci are engulfed and killed by antigen presenting cells, bacterial proteins are digested and presented by the major histocompatibility complex II (MHC-II) on the surface of antigen presenting cells. These antigen peptide/MHC-II-complexes are then recognized by specific lineages of T-cells which become activated upon binding. This leads to clonal expansion of immune cells capable of mediating a specific antibody or cellular immune response directed against the invading microorganism.

So far, the exact mechanisms for the involvement of T-cells in pneumococcal infections are not fully understood. However, $CD4^+$ T-cells are important in establishing and maximizing the capabilities of the adaptive immune response. These cells can not kill infected cells or clear pathogens since they have no phagocytic or cytotoxic activity. Instead they possess a managing role in the immune response by directing other cells to perform these tasks. Kemp *et al*. investigated *in vivo* the levels of activated $CD4^+$ T-cells producing type 1 cytokines in the circulation. They found that activated T-cells with a type 1 cytokine profile decreased early in the acute phase of the pneumococcal infection suggesting that these cells are highly engaged in the *in vivo* immune response to pneumococci (Kemp *et al.* 2002). The underlying mechanisms for this disappearance may be increased apoptosis and/or sequestration in the peripheral tissues.

Adaptive immunity to extracellular bacteria, such as *S. pneumoniae*, is largely conferred by antibodies. Pneumococci are rapidly cleared from the blood, mainly by the liver and to a lesser extent by the spleen, in the presence of anti-capsular antibodies, however, complement is required to achieve effective clearance (AlonsoDeVelasco *et al.* 1995). Immunoglobulin responses specific for pneumococcal protein and polysaccharide antigens are induced through distinct mechanisms (Mond *et al.* 1995). Capsular polysaccharide antigens elicit antibodies that are isotype restricted to IgM and IgG_2 and in a lesser extent to IgG_1 in humans, and IgM and IgG_3 in mice (AlonsoDeVelasco *et al.* 1995). Polysaccharides are unable to recruit cognate $CD4^+$ T-cell help through T-cell receptor recognition of peptide/MHC-II complexes on the surface of antigen presenting cells. Instead, the regularly spaced repeating epitopes expressed by polysaccharides induce multivalent membrane immunoglobulin cross-linking on the B-cell surface leading to only B-cell proliferation. Therefore, capsular polysaccharides yield no anamnestic response and are weakly immunogenic in children under two years of age. Although polysaccharides fail to associate with MHC-II, their co-expression with proteins by an intact microbe could mediate cognate $CD4^+$ T-cell help for polysaccharide-specific B-cells. The immune response to T-cell dependent antigens critically depends on the interaction between CD40 ligand on the surface of activated $CD4^+$ T-cells, and CD40 expressed on the surface of B-cells. This interaction stimulates the resting B-cell to secrete antibodies to T-cell-dependent antigens. During an *in vivo* response to intact pneumococci, the blocking of CD40-CD40 ligand interactions inhibited induction of immunoglobulin specific for capsular polysaccharides (Hwang *et al.* 2000).

Hitherto, the impact of innate cytokine release on the subsequent development of the adaptive antibody response to an extracellular bacterium is not fully understood. There are, however, studies of tumor necrosis factor, a proinflammatory cytokine, which strongly suggest a link between cytokines released during the early, innate response and the subsequent development of adaptive humoral immunity (AlonsoDeVelasco *et al.* 1995). The quality and strength of the humoral response appears to be balanced by the concomitant secretion of a panel of cytokines having opposing actions, and which regulate differentially *in vivo* polysaccharide- and protein-specific immunoglobulin response to *S. pneumoniae* (Khan *et al.* 2002).

1.6.2 Immune response to vaccines

Currently there are two different pneumococcal vaccine formulations, polysaccharide vaccine and conjugate vaccine, available on the market. These two vaccine types differ in immunogenicity. Polysaccharide vaccines are based on extracted and purified forms of the bacterial polysaccharide capsule. The rationale behind these purified polysaccharide vaccines is that they would trigger the production of circulating antibodies, which then would cover the bacterial capsules with antibodies and/or complement, promoting phagocytosis and removal by cells (e.g., macrophages and neutrophils) of the innate immune system. However, purified polysaccharides cannot be presented via MHC-II for $CD4^+$ T-cells. Instead they activate B-cells in a T-cells independent manner by cross-linking antibodies on the B-cell surface, which results in the formation of plasma B-cells and antibodies without the help of $CD4^+$ T-cells (Griffioen *et al.* 1992). Consequently, IgM is the predominant isotype generated due to little class switching, no affinity maturation, and little development of memory cells. Vaccines composed solely of purified polysaccharides are only effective in older children and adults for a period of three to five years. They are not effective for infants and very young children under two years of age, who have immature immune systems (Cowan *et al.* 1978). Therefore, conjugate vaccines have been developed. These protein-polysaccharide vaccines link purified bacterial polysaccharides to purified protein carrier, which involve $CD4^+$ T-cells in the polysaccharide antigen response. The protein carrier of the conjugate vaccine leads to antigen presentation on antigen presenting cells. The interaction between B-cells and $CD4^+$ T-cells permit class switching from IgM to IgG and affinity maturation of antibody, which makes plasma cells more specific, longer-lasting effectors. Memory cells are also induced to participate in a secondary response upon exposure to the pathogen. Several protein carriers have been used in conjugate vaccine strategies, for example tetanus toxoid, diphtheria toxoid, and a non-toxic variant of diphtheria toxin referred to as CRM_{197}. These protein carriers were all selected on the basis of their capacity to activate $CD4^+$ T-cells (AlonsoDeVelasco *et al.* 1995). In pneumococcal vaccines, pneumococcal-CRM conjugates have been most extensively studied. Each of the included polysaccharides is in that case coupled to CRM_{197}. More about pneumococcal vaccines and their effects is to be found in section 1.8.6.

1.7 EPIDEMIOLOGY

1.7.1 Incidence

Pneumococcal disease is endemic all over the world. In 2002, World Health Organization (WHO) estimated that approximately 1.6 million deaths occurred around the world as a result of pneumococcal disease (2005). Pneumococcal pneumonia alone is responsible for more than one million deaths annually, making it one of the leading infectious causes of mortality. Around half of the deaths caused by pneumococcal diseases occur in young children (<5 years), mainly in developing countries. In the developed world, pneumococcal infection is more common as a cause of death among the elderly than in young children.

Mucosal infections are usually less severe but much more common than invasive infections (Figure 4). In Sweden, the pneumococcus is estimated to annually account for between 30,000 and 70,000 cases of AOM (Läkemedelsrådet 2009), and around 80,000 cases of sinusitis (Läkemedelsrådet 2009).

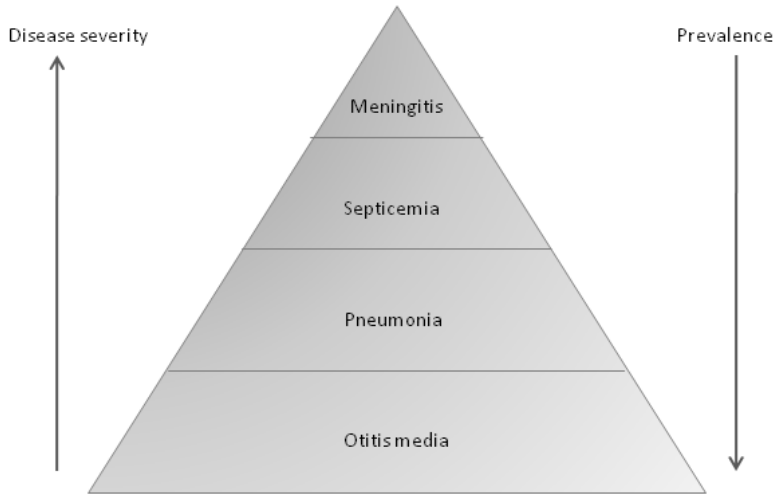

FIGURE 4 For the most common diseases caused by pneumococci, there is an inverse relationship between disease severity and prevalence. Adapted from Edwards, KM., Pneumococcal Infections: Therapeutic Strategies and Pitfalls. In *The Pneumococcus*. (2004) ASM Press, Washington D.C.

Concerning pneumonia, *S. pneumoniae* dominates as an etiologic agent of CAP in all age groups and is probably responsible for nearly half of the cases (Iwarson-Norrby 2007). The yearly incidence of CAP for adults is around 1% but increases with age (Iwarson-Norrby 2007). For young children below five years of age the corresponding incidence is higher, between 3-4%, whereas among children 5-14 years the incidence is

1-2% (Iwarson-Norrby 2007). The corresponding figures in rest of Europe and in the United States are roughly the same.

The incidence of IPD seems to vary substantially by age, ethnicity, geographic location, socioeconomic status, and immune status (van der Poll *et al.* 2009). In isolated indigenous populations, such as Aboriginal Australians, the Maoris of New Zealand, and Alaskan Eskimos, the yearly incidence rates of IPD among children have been reported to be as high as 441 cases per 100,000 individuals (Bruce *et al.* 2008; Voss *et al.* 1994). According to WHO, the annual incidence for pneumococcal bacteremia in Western populations is between 15-19 cases per 100,000 individuals, whereas the corresponding number for meningitis is 1-2 (WHO 2003). In Sweden, the total annual incidence of pneumococcal meningitis is at least 1 per 100,000 individuals, whereas of pneumococcal bacteremia it is around 15 per 100,000 (Giesecke *et al.* 1997). During 2009, the reported IPD incidence was 17 per 100,000 individuals (www.smi.se; Swedish Institute for Infectious Disease Control). The incidence of pneumococcal infections shows seasonal variation and is highest during the winter months; in Sweden the majority of the cases occur during the period from October to April.

Even in economically developed regions, the mortality rate for IPD is high. For example, studies in the United States have reported about a case-fatality rate for IPD between 20-40% (Austrian 1964; Hook *et al.* 1983; Mufson *et al.* 1982; Tomasz 1997). In Sweden, the case-fatality rate for pneumococcal pneumonia is 7-8%, whereas for IPD it averages 20-30% (Burman *et al.* 1985; Örtqvist 1999). Even in Western populations, the mortality rate may exceed 50% in certain high-risk groups (WHO 2005).

1.7.2 Risk factors associated with pneumococcal disease

1.7.2.1 Age

A major risk factor for acquiring a pneumococcal disease is age. The incidence is highest in individuals at the extremes of age, including children younger than two years and adults over 65 years of age. The increased risk of disease among children younger than two years of age is probably due to immature immunological response to the polysaccharide capsule and also a high colonization rate. Among elderly, the higher rates of underlying medical conditions and age-related immune dysfunction contribute to the higher rates of pneumococcal infection and death (Castle 2000).

1.7.2.2 Sex, race, and ethnicity

Among people with pneumococcal disease, males are overrepresented among infants, children and adults (Klein 1981; Robinson *et al.* 2001). Also certain racial and ethnic groups seem to be at higher risk for acquiring pneumococcal disease. In Australia for example, the rates of IPD among Aborigines are between 4 to 30 times those reported for non-Aborigines (Roche *et al.* 2008; Torzillo *et al.* 1995; Trotman *et al.* 1995). Similarly, in the United States rates of IPD are higher for blacks and Native Americans than for whites and for persons of other racial backgrounds (Cortese *et al.* 1992; Pastor *et al.* 1998; Robinson *et al.* 2001; Rudolph *et al.* 2000). Contributing factors to these disparities in disease rates are inadequately examined, but may involve differences in living conditions and in prevalence of immunocompromising conditions. High rates of HIV infection among blacks in urban areas have been suggested as an important determinant, although among HIV-infected persons the risk of IPD is higher for blacks than for white (Dworkin *et al.* 2001; Gebo *et al.* 1996).

1.7.2.3 Chronic medical conditions

For persons with certain chronic medical conditions, for example HIV infection, asthma, cirrhosis, diabetes mellitus, functional or anatomic asplenia, chronic renal failure, hematological malignancies, and for persons taking immunosuppressive medications, the risk and severity of pneumococcal infection is increased (Burman *et al.* 1985; Lipsky *et al.* 1986).

1.7.2.4 Living conditions

Most pneumococcal infections occur sporadically despite the seasonal pattern, i.e., they are not a part of a recognized disease outbreak or cluster. However, occasionally there are reports about outbreaks of pneumonia, meningitis, and conjunctivitis caused by a single pneumococcal strain. Majority of the outbreaks occur in institutional settings such as jails (Hoge *et al.* 1994), hospital wards and care facilitates (Gleich *et al.* 2000; Nuorti *et al.* 1998; Subramanian *et al.* 2003), military units (Gray *et al.* 1999), homeless shelters (Mercat *et al.* 1991), and day-care centers (DCCs) (Cherian *et al.* 1994; Craig *et al.* 1999). A common factor in these pneumococcal outbreaks is crowding which facilitates transmission of the organism to many susceptible persons.

Another risk factor in living conditions seems to be the frequency of contact with preschool children. Day-care attendance has for example been strongly associated with an elevated risk of IPD for children, and exposure to preschool children appears to

increase the risk of pneumococcal disease in adults (Hendley *et al.* 1975; Levine *et al.* 1999; Takala *et al.* 1995).

1.7.2.5 Alcohol and tobacco use

Heavy alcohol use and cigarette smoking have been observed to be common among otherwise healthy persons with pneumococcal disease and appears to be an important risk factor (Burman *et al.* 1985; Lipsky *et al.* 1986). Likewise, a study of immunocompetent adults between 18 to 64 years of age, reported that more than half of all cases of IPD were attributable to cigarette smoking (Nuorti *et al.* 2000).

1.7.2.6 Preceding or coincident infection

During the winter months in temperate areas when respiratory viral infections are most common, pneumococcal infections are more common (Dowell *et al.* 2003). In children, AOM often develops within days after onset of upper respiratory tract infections (Heikkinen *et al.* 2003), and adults with IPD is reported to have upper respiratory tract infections during the preceding month at a higher rate, compared with age-matched controls (Nuorti *et al.* 2000). There are also several lines of epidemiological evidence suggesting a relationship between influenza A virus and pneumococcal disease (Kim *et al.* 1996; O'Brien *et al.* 2000). Adults who have been experimentally infected with influenza A virus demonstrate increased susceptibility to nasopharyngeal colonization with pneumococci (Wadowsky *et al.* 1995).

1.7.3 Molecular epidemiology

In recent years, the use of molecular strain-typing methods has become increasingly integrated into the pneumococcal epidemiology. Examination of the molecular epidemiology of pneumococci gives deeper insights into spreading patterns and may help to develop and establish infection control measures. For example, molecular epidemiological studies of pneumococci have reported that a few clones (i.e., closely related isolates) are particularly successful in the global spread, being isolated over several years and contributing significantly to pneumococcal disease. Besides, the same clones are found in the nasopharynx of healthy children (Enright *et al.* 1999; Enright *et al.* 1998; Hermans *et al.* 1997; McGee *et al.* 2001).

The major reasons for epidemiological surveillance are the increasing rates of resistant pneumococcal isolates over the world as well as the high incidence in pneumococcal infections. Both phenotypical and genotypical methods have been

developed and applied in epidemiological studies of pneumococci. For a long time, phenotypical approaches such as serotyping and antibiotic susceptibility profiles were the only techniques available to discriminate different pneumococcal strains. These methods are still very valuable, but have generally poor discriminatory power between clones. Therefore, several typing techniques based on genetic differences have been developed and applied in pneumococcal epidemiology. Examples of some methods used are various DNA fingerprinting methods such as ribotyping (Cherian *et al.* 1994; Kell *et al.* 1993; McDougal *et al.* 1992), DNA fingerprinting of the penicillin-binding proteins (PBPs) (Kell *et al.* 1993; Munoz *et al.* 1991; Munoz *et al.* 1992; Smith *et al.* 1993), BOX fingerprinting (Hermans *et al.* 1995; Martin *et al.* 1992), pulsed-field gel electrophoresis (Lefevre *et al.* 1993; Lefevre *et al.* 1994), and restriction fragment length polymorphism (Hermans *et al.* 1995; van Steenbergen *et al.* 1995). The most recently developed method is multi-locus sequence typing which is based on the sequencing of around 450 bp internal fragments of seven housekeeping genes, resulting in a higher level discriminatory even by using a few numbers of loci (Enright *et al.* 1998; Hanage *et al.* 2005).

1.8 PREVENTION AND TREATMENT

1.8.1 Antibiotic resistance

The historically significant discovery of penicillin for almost hundred years ago revolutionized the medical treatment of patients since it was effective against many previously very serious infectious diseases. The widespread use of penicillin resulted in a dramatically reduction of the morbidity and mortality caused by various bacterial agents, including *S. pneumoniae*. Treatment options for pneumococcal infections are dependent on the site of infection and the degree of intrinsic drug resistance. For a long time, penicillin has been the drug of choice for all pneumococcal diseases because the bacterium was sensitive to antibiotic concentration easily achievable by either the oral or the parenteral course. However, during the last decades the therapy of pneumococcal infections has been complicated by the rapid emergence of antibiotic-resistant pneumococci. Examples of other antimicrobial agents used for pneumococcal infections include erythromycin, clindamycin, TMP-SMX, tetracycline, and vancomycin.

However, already in 1967 the first penicillin resistant pneumococcal isolate was reported in Australia (Hansman 1967). Ever since then, the frequency of resistant

isolates has increased dramatically, and today penicillin resistant pneumococci and penicillin non-susceptible pneumococci (PNSP) have been identified all over the world. Also resistance to other antibiotics occurs among pneumococci, often in combination with penicillin resistance. The prevalence of multidrug-resistant pneumococci is increasing. In some countries, including the United States, Spain, France, South Africa, Israel and Japan, over 25% of the collected pneumococcal isolates are multi-resistant (Jacobs *et al.* 2003). It should be stressed that the definition of pneumococcal multi-resistance varies between studies so making a comparison of proportions is difficult. According to Swedish Institute for Infectious Disease Control (www.smi.se), multi-resistant pneumococci are defined as isolates resistant to penicillin and at least two other antibiotics, whereas the Centers for Disease Control and Prevention (or CDC; the United States government public health agency) defines multidrug-resistance to include any isolate exhibiting resistance two or more of the following classes of antibiotics: second-generation cephalosporins, macrolides, penicillins, tetracyclines, and sulphonamides (Thornsberry *et al.* 2008). However, multi-drug resistance is a serious clinical concern since it may limit the treatment possibilities considerably.

In Sweden, the prevalence of resistant pneumococci is still a relatively minor problem in most regions. Only 3-6% of strains demonstrate decreased susceptibility to penicillin (**paper IV**; Borres *et al.* 2000; Molstad *et al.* 2008; Örtqvist 1999). However, certain regions such as the Stockholm area and southern Sweden suffer from higher prevalence of up to 10% of PNSP (Ekdahl *et al.* 1994). Also for other antibiotics, the resistance situation seems rather favorable; around 2-4% of the strains have been reported resistant to macrolides, 2% to tetracyclines, and 4-6% to trimethoprim-sulphametoxazole (TMP-SMX) (**paper IV**; Örtqvist 1999).

1.8.2 Emergence and spread of resistance in pneumococci

A key role in determining how resistance emerges and spreads is the genetic basis (Figure 5). Several biological characteristics distinguish pneumococci from many other pathogens with acquired resistance. First, the primary source for pneumococcal spreading is asymptomatically carriage in the nasopharynx which may harbor both sensitive and resistant strains. Second, *de novo* resistance in pneumococci is rarely the result of single point mutation or plasmid carriage. Examples of important exceptions are TMP-SMX resistance and low-level fluoroquinolone resistance in which resistance is conferred by single point mutations in clinical isolates. However, resistance to the most major classes of antibiotics is acquired either by transformation or transfer via

conjugative transposons. Besides, the degree of resistance to certain antibiotics can vary widely in resistant strains. This graded phenomenon is measured as the minimum inhibitory concentration (MIC) of antibiotic necessary to inhibit growth, where different MICs are associated with different genetic alterations. Such biological features directly influence the population dynamics of pneumococcal resistance.

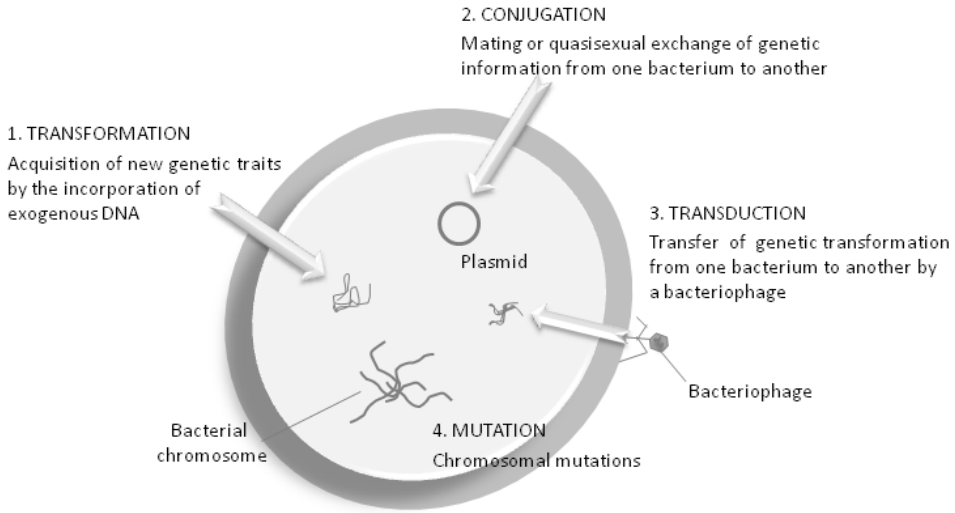

FIGURE 5 Mechanisms for acquisition of antibiotic resistance determinants. Chromosomal mutations that reduce the sensitivity of the antimicrobial targets are common mechanism of resistance to several antimicrobials such as β-lactams, quinolones, and TMP. The horizontal spread of resistance to these antibiotics into different pneumococcal strains is probably due to genetic transformation, whereas the resistance determinants for macrolides and tetracycline are typically borne on conjugative transposons which can be transferred either intra- or intercellularly.

Asymptomatic carriers of pneumococci contribute more to pneumococcal spread than symptomatic individuals, in contrast to infectious diseases that do not include a carrier state. For most of the major antimicrobial classes, resistant pneumococci are spread mainly by clonal amplification. This means that infections with resistant pneumococci primarily are the result of acquisition of resistant strains from asymptomatic carriers in the community. Consequently, the major determinant for the global emergence of antibiotic resistance has been found to be the successful spread of a few resistant pneumococcal clones (Richter *et al.* 2002; Sjostrom *et al.* 2007).

Interestingly, most clinical isolates associated with antimicrobial resistance belong to serotypes 6B, 9V, 9A, 14, 19F, and 23F (Schrag *et al.* 2000). It is still unclear why these serotypes have a higher probability of containing resistance determinants. However, a plausible explanation is that these serotypes are commonly isolated from children and thereby carried for longer durations and thus exposed to increased

antibiotic pressure. Besides, transformation may play an important role in acquiring horizontally spread resistance determinants. Hitherto, the proportion of clinical isolates that are transformable in the laboratory has not been defined. Various capsular types may reduce or totally block transformation. Perhaps, differences in the competence regulon may limit the acquisition of resistance to particular clonal groups.

1.8.3 Population-biological considerations

Antibiotic therapy affects the pneumococcal population biology in several ways. The direct effect is mediated by inhibiting or killing those pneumococci that are susceptible to the drug at the achieved concentration. In a treated individual, the antibiotic-mediated inhibition of susceptible strains will promote the growth of resistant strains given that pneumococcal strains compete to colonize nasopharynx (Lipsitch 2001b). Even if no resistant strain is present in the treated individual, inhibition of susceptible strains in an antibiotic-treated person could promote the spread of resistant strains in the community by reducing transmission of the susceptible strains with which the resistant ones compete (Lipsitch *et al.* 2002). Any used antibiotic that inhibits susceptible strains more than resistant ones will exert natural selection in favor of the strains against which it is less active. The relative efficacies against the susceptible and the resistant strains determine the degree of selection.

Numerous epidemiologic studies have recognized recent antibiotic use as the strongest risk factor for the carriage and spread of non-susceptible *S. pneumoniae* at both community (Arason *et al.* 1996; Kristinsson 1995; Melander *et al.* 2000) and individual levels (Dagan *et al.* 1996; Dowell *et al.* 1997). In several countries, efforts to control antibiotic consumption have been undertaken in hopes of slowing the rising resistance trends in microorganisms. An important determinant in evaluating the effects from such efforts is the extent to which non-susceptible strains have any biological cost in competition with fully susceptible strains. That means, if no such fitness cost exist there is no disadvantage to being resistant and then there will be no selective pressure to drive down the rate of resistant strains even if antibiotic use were reduced to zero (**paper II**; Lipsitch 2001c). Moreover, in other microbes it have been observed that compensatory mutations occur which counteract the deleterious effects of resistance (Andersson 2003; Lipsitch 2001c). Hitherto, few studies have investigated this for clinically important resistance mechanisms in *S. pneumoniae* (Gillespie *et al.* 2002; Rieux *et al.* 2001). The successful global spreading of multi-resistant pneumococcal strains suggests, however, that if such fitness costs exist, they may be offset in certain

clones by association with other genetic features that enhances the fitness (Sjostrom *et al.* 2007). A simple reduction of the antibiotic consumption in the community may slow down the increase in resistance in pneumococci, but other interventions appears to be required to decrease the absolute level of resistance, for example large-scale vaccine programs (**paper II**). According to a previous modeling study, one of the most efficient ways to reduce pneumococcal transmission in the community is to reduce the group sizes in the DCCs (**paper I**). Such an intervention will most likely also result in fewer communicable diseases among children attending DCCs and thus, less antibiotic prescription.

1.8.4 Mechanisms of resistance

The emergence of antibiotic resistance observed in pneumococci all over the world during the last decades reflects dissemination of a few clones. The resistance rates vary significantly among different geographic regions and are influenced by patterns of antibiotic consumption, population density, and local prevalence of resistant strains. In this section, the mechanisms of action and resistance for some of the antibiotics often used for treating pneumococcal infections are described. Figure 6 summarizes the most common mechanisms mediating antibiotic resistance in bacteria.

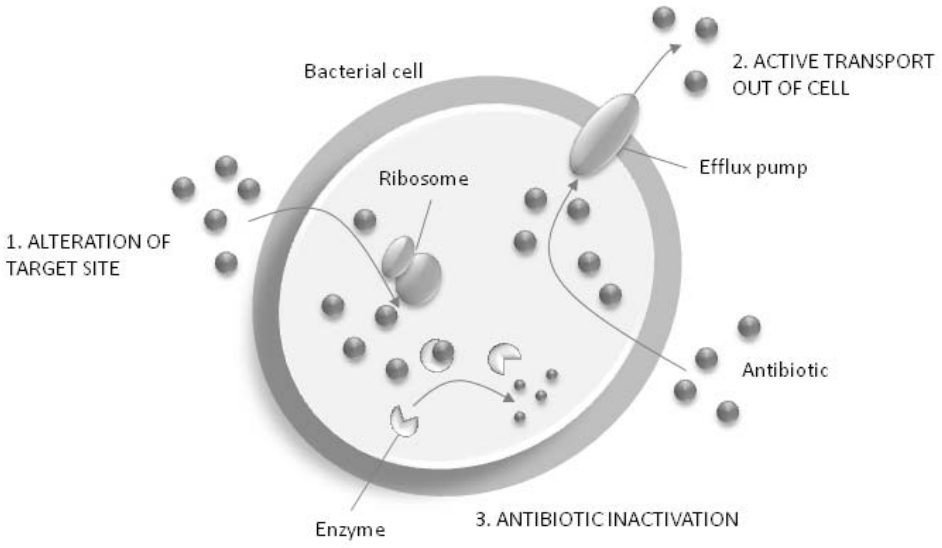

FIGURE 6 Overview of the most common resistance mechanisms in bacteria. Alteration of target sites can for example be methylation of the bacterial ribosomes via enzymes. Another mechanism is active transport of antibiotics out of the bacterial cell via efflux pumps, whereas inactivation of antibiotics can occur via degrading or altering enzymes.

1.8.4.1 Penicillin

The most common mechanism of antibiotic activity is interference with bacterial cell wall synthesis. A majority of the cell-wall inhibiting antimicrobials are classified as β-lactam antibiotics (e.g., penicillin, cephalosporin, cephamycins, β-lactamase inhibitors), called so because they share a β-lactam ring structure. The target proteins for β-lactams are bacterial enzymes that catalyses late steps in murein biosynthesis. Ever since the discovery of penicillin, it has been the drug of choice for pneumococcal infection. The popularity of penicillin is due to its high efficacy combined with an extremely low toxicity. However, the emerged penicillin resistance in pneumococci as well as in other pathogens has had a major impact on treatment regimes. The simplest mechanism for resistance against penicillin and other β-lactams is the production of β-lactamases. These enzymes are frequently encoded on plasmids and can therefore be readily transmitted between bacteria. Although pneumococci are naturally transformable, no β-lactamase-producing isolate has been recognized to date (Klugman 1990). Instead, penicillin resistance in pneumococci is mediated by the alteration of penicillin target enzymes known as PBPs. These membrane-associated enzymes are believed to catalyze the terminal stages of murein synthesis and are inhibited by bonding with penicillin at their active site. The binding of penicillin to PBPs inhibits the cell wall synthesis and activates the LytA autolysin. Alterations in PBPs of resistant strains lead to decreased affinity to the drug so that higher penicillin concentrations are required for binding and inhibition of the enzyme (Hakenbeck *et al.* 1999b). There are six variants of PBPs found in susceptible strains: PBP1a, PBP1b, PBP2x, PBP2a, PBP2b, and PBP3. All six PBPs can occur as low affinity variants. Restructuring of the PBPs confers resistance; the level of resistance is determined by how many and to what extent the targets are modified. Several mutations in PBP2x or PBP2b lead to low affinity binding of β-lactams, providing a low-level resistance. These low-affinity forms of PBP2x and PBP2b are prerequisites for high-level β-lactam resistance as they results in high-level resistance in combination with low affinity variants of PBP1a (Hakenbeck *et al.* 1999b).

In resistant clinical isolates of pneumococci, low-affinity PBPs are encoded by mosaic genes in which the PBP gene sequences are replaced by allelic variants that may differ by up to 25% in DNA sequence. It is believed that resistance has evolved in closely related streptococci via acquisition of point mutations in PBP genes prior to the transfer into *S. pneumoniae*. Genetic competence has obviously provided a valuable tool for the acquirement of resistance determinants that have evolved within the larger

streptococcal community gene pool. Studies have revealed that at least three PBPs are encoded by mosaic genes in high-level resistant clinical isolates: PBP1a, PBP2b, and PBP2x (Hakenbeck *et al.* 1999a).

A phenomenon called penicillin tolerance has also been described in pneumococci (Tomasz *et al.* 1970). A tolerant strain has the ability to survive during therapy, but does not replicate and the growth is resumed after removal of the drug. Resistant strains seem to evolve more easily from tolerant ones without losing too much in fitness. It has, for example, been demonstrated that a penicillin tolerant strain could be transformed to resistant in one single step, in contrast to the non-tolerant parental strain (Novak *et al.* 1999). Also tolerance against vancomycin has been reported for pneumococcal strains (Gillis *et al.* 2005; Henriques Normark *et al.* 2001; Hidalgo *et al.* 2003; Sung *et al.* 2006). This is alarming since such strains could potentially develop into resistant ones (Novak *et al.* 1999). However, the exact background of tolerance has not been fully understood. From a clinical perspective, tolerance may lead to problem since the bacteria are not killed during therapy and will continue to grow as soon as the treatment is terminated. Besides, it is difficult to detect tolerant bacteria since they have the same MIC as susceptible strains.

Ever since the first report about penicillin resistant in a clinical isolate of pneumococci in 1967 (Hansman 1967), the frequency of resistant isolates has increased and the prevalence varies widely between geographic regions. For example, a study including eight European countries reported penicillin resistance rates ranging from 4.4% (Austria) to 61.9% (Spain) (Reinert *et al.* 2005). An Asian study including 12 countries revealed an average penicillin resistance rate of 29.4% where isolates from Vietnam showed the highest prevalence of penicillin resistance (71.4%) (Song *et al.* 2004). According to population-based invasive pneumococcal surveillance during 2007 in the United States, around 26% of the pneumococcal isolates were reported as PNSP (Gertz *et al.* 2010). As mentioned previously, studies performed in Sweden have reported relatively low rates of pneumococci with reduced susceptibility against penicillin (**paper IV**; Borres *et al.* 2000; Ekdahl *et al.* 1994; Molstad *et al.* 2008; Örtqvist 1999).

1.8.4.2 Erythromycin

The antimicrobial agent called erythromycin belongs to the group of macrolides which have a common structure formed by a large lactone ring. Erythromycins exert their effect by reversibly bind to the 23S rRNA of the bacterial 50S ribosomal subunit,

thereby blocking the polypeptide elongation during bacterial protein synthesis. In *S. pneumoniae*, the resistance to erythromycin is due to modification of the ribosomal target by methylation or mutation or by active efflux of the antibiotic. Ribosomal modification by methylation was the first resistance mechanism to erythromycin that was elucidated. It is secondary to the acquisition of an *erm* gene which is usually carried by transposable elements in pneumococci. This ribosomal methylase dimethylates 23S rRNA at a single site, adenine at position 2058, thereby causing a markedly reduced affinity of erythromycin for its target. Cross-resistance to macrolides, lincosamides, and streptogramin B antibiotics, referred to as MLS_B-phenotype, is caused by the overlapping binding sites of the antibiotics. Expression of MLS_B-type resistance can be either constitutive or inducible. Even if *erm*(B) is widely predominant, it is not the only representative of the *erm* gene class in pneumococci. For example, the presence of *erm*(A), initially designated *erm*(TR), has been reported in rare cases (Syrogiannopoulos *et al.* 2001).

Erytromycin resistance in pneumococci mediated by active efflux is usually caused by the presence of the *mef*(A) determinant. The *mef*(A) gene encodes an efflux pump that results in resistance only to 14- and 15-membered-ring macrolides, so called M-phenotype. Isolates harboring the *mef*(A) gene normally display less marked resistance to erythromycin, whereas isolates with the *erm*(B) determinant usually show a high-level resistance. Other macrolide resistance mechanisms detected in pneumococci include mutations in a highly conserved region of domain V of 23S rRNA and in ribosomal proteins L4 and L22 (Canu *et al.* 2002; Tait-Kamradt *et al.* 2000a; Tait-Kamradt *et al.* 2000b).

The first erythromycin resistant isolates of *S. pneumoniae* was reported in 1967 in the United States and subsequently worldwide (Klugman 1990; Weisblum 1967). Although macrolide resistance in pneumococcal strains is prevalent, it varies markedly in different countries (Pallares *et al.* 2003). For example, in France and Italy over 40% of isolates have been found resistant (Schito *et al.* 2000), in contrast to only ~3% of strains in Norway (Littauer *et al.* 2005) and Sweden (**paper IV**). The distribution of the two dominating resistance types, *erm*(B) and *mef*(A), varies, but MLS_B is more prevalent in Europe (Montanari *et al.* 2001) whereas the efflux type predominates in the United States and Canada (Johnston *et al.* 1998).

1.8.4.3 Clindamycin

Clindamycin is a lincosamide antibiotic and its mechanism of action resembles that of macrolides. This agent binds to the 23S rRNA subunit of the bacterial ribosome and prevents translocation of the peptidyl-tRNA from the A-site to the P-site during protein synthesis. The ribosome therefore releases an incomplete protein. Pneumococcal isolates carrying the *erm*(B) gene, and thereby exhibiting the MLS_B-resistance phenotype, are resistant to clindamycin. As described previously, the *erm*(B) encoded methylase alters the ribosomal binding site which confer high-level cross-resistance to macrolides, lincosamides, and streptogramin B antibiotics.

1.8.4.4 Trimethoprim-sulphametoxazole

Sulphonamides (SMX) were the first class of antimicrobial agents introduced into clinical use, which occurred in 1935. Trimethoprim (TMP) was introduced in 1962, and the combination TMP-SMX was brought into clinical use in 1968. These two agents act synergistically to inhibit enzyme systems involved in the bacterial synthesis of tetrahydrofolic acid. Resistance to TMP-SMX in a clinical pneumococci isolate was first identified in 1972 (Howe *et al.* 1972). Today, TMP-SMX resistance is very commonly found among pneumococci. Rates of TMP-SMX resistance appears to be higher among carriers than in clinical isolates (Klugman 1990). For example, TMP-SMX resistant strains collected from healthy carriers in Spain comprised 79.2% of isolated pneumococci (Perez *et al.* 1987), whereas in clinical specimens between 37.7 - 51.7% of the strains showed TMP-SMX resistance (Klugman 1990; Perez *et al.* 1987). Sweden appears to be a region with relatively low prevalence of TMP-SMX resistant pneumococci; reported rates range from 1.7% to 4% among clinical isolates (**paper IV**; Zackrisson *et al.* 1981). Resistance to TMP in pneumococcal isolates is mediated by multiple mutations in a gene encoding a reductase enzyme, while isolates with reduced susceptibility carry a single mutation (Maskell *et al.* 2001). Similarly, altered chromosomal reductase genes can lead to resistance to sulfonamides. It has been shown that isolates which are resistant to TMP rarely are sensitive to TMP-SMX (Adrian *et al.* 1997). Hence, TMP-SMX appears to correlate more strongly with TMP resistance than SMX resistance.

1.8.4.5 Tetracycline

In 1948, tetracycline antibiotics were introduced into medicine and were then quickly accepted since they offered a broad spectrum of activity. The action of tetracycline is

presumed to be mediated by binding to a site on the bacterial 30S ribosomal subunit and thereby inhibiting the elongation phase of protein synthesis (Connell *et al.* 2003a). In *S. pneumoniae*, tetracycline resistance is a result of acquisition of one of two resistance determinants, *tet(M)* or *tet(O)*. These genes encode ribosomal protection proteins. Thus far, ribosomal protection is the only mechanism for tetracycline resistance described. It appears that Tet(M) and Tet(O) confer resistance by promoting the release of the drug from its inhibitory site on the ribosome (Burdett 1996; Connell *et al.* 2003b). The most commonly found tetracycline resistance gene in pneumococci, *tet(M)*, is located on two transposons called Tn*1545* and Tn*5253*, whereas the *tet(O)* gene, which is chromosomally situated, probably have been acquired trough transformation (Widdowson *et al.* 1998). In streptococci, *tet(O)* can be conjugatively transferred on plasmids to other Gram-positive bacteria; however, plasmids are not common in pneumococci (Widdowson *et al.* 1998).

1.8.4.6 Fluoroquinolones

Fluoroquinolones acts by interfering with bacterial DNA metabolism through inhibition of two essential bacterial DNA topoisomerases, DNA gyrase and topoisomerase IV. These enzymes are involved in the regulation of chromosome supercoiling and decatenation. A cleavable complex is formed between quinolones and DNA topoisomerases which results in irreversible double-strand DNA breaks, leading to cell death (Drlica *et al.* 1997; Khodursky *et al.* 1998). Fluoroquinolone resistance involves two main mechanisms, target modification and increased active efflux. Generally, low-level resistance is caused by mutations in the quinolone resistance-determining region in either of the target genes, whereas high-level resistance requires additional mutations in the quinolone resistance-determining region of the second target (Pan *et al.* 1996; Pan *et al.* 1997; Pan *et al.* 1998). In clinical isolates of pneumococci, quinolone resistance is primarily due to mutated subunits C of topo IV and/or subunits A of gyrase (Varon *et al.* 2000). The other phenotype of resistance is caused by an increased active efflux of the drug mediated directly by adenosine triphosphate hydrolysis or by the proton motive force, which confer a moderate level of resistance to levofloxacine, norfloxacine, and ciprofloxacine (Varon *et al.* 2000).

Ciprofloxacine has been in widespread clinical use for bacterial diseases over a decade; however, because of its low activity against Gram-positive bacteria it is not an ideal antibiotic for respiratory infections. Newer quinolones (e.g., gatifloxacin, gemifloxacin, and moxifloxacin) have shown an enhanced activity against the majority

of respiratory pathogens and are therefore more widely used. There are studies reporting about rapidly emerging frequency of quinolone resistance in pneumococci (Deshpande *et al.* 2006; Pallares *et al.* 2003), whereas others, in contrast, report about a low prevalence of quinolone resistant strains (Davies *et al.* 2008; Pletz *et al.* 2010).

1.8.4.7 Vancomycin

Vancomycin acts by inhibiting the synthesis of the bacterial cell wall. For sensitive bacteria, exposure to vancomycin leads to arrest of bacterial growth. At the same time, autolysins are activated and subsequently, cell lysis occurs. Vancomycin is used more frequently to treat severe, invasive infections caused by multidrug-resistant pneumococci. Although no vancomycin-resistant isolates have been recognized hitherto, vancomycin-tolerant pneumococcal strains failing to die rapidly and thereby causing relapsing disease have been reported over the world (Gillis *et al.* 2005; Henriques Normark *et al.* 2001; Hidalgo *et al.* 2003; Sung *et al.* 2006). Strains that do not undergo autolysis after treatment with vancomycin show an increased survival and are termed tolerant. The exact vancomycin tolerance mechanism in pneumococci remains to be determined, however, it appears to be a multifactorial phenomenon arising from the effects of the bacterial capsule (Fernebro *et al.* 2004), the autolysin LytA (Tomasz *et al.* 1970), and the ABC transport system Vex123 (Haas *et al.* 2005; Haas *et al.* 2004).

1.8.5 Clinical relevance of resistance

The clinical relevance of antibiotic resistance *in vitro* among pneumococci is still not fully elucidated despite increasing resistance rates over the world. There is a debate whether increasing resistance to β-lactams, macrolides, and other antimicrobial agents has been followed by increased rates of treatment failure and mortality. Although rising resistance rates, these agents are still used as first-line empirical therapy in the outpatient setting. A reason for this may be the infrequency with which clinicians recognize clinical failures. It appears like the present *in vitro* definitions of drug resistance in pneumococci are not appropriate for all types of pneumococcal infections. The clinical failures observed with the emergence of resistance to some classes of antimicrobials and the successful continued use of other agents despite emerging resistance can be explained by the use of pharmacodynamics. The impact of resistance on bacterial eradication and clinical outcome can be examined by measuring antimicrobial susceptibility (i.e., MIC) and compare it to the achievable unbound

fraction of drug at the infection site. Thereby, measures of the ability of the antibiotic to kill or inhibit bacterial growth are obtained (Craig 1998).

To most clinically important antibiotics classes, most pneumococcal resistance is high-level resistance. This means that the MICs for resistant strains are well above clinically achievable concentrations. A major exception is, however, pneumococcal resistance to penicillin. The definition for PNSP is a MIC-value ≥0.125 μg/ml (www.smi.se) whereas a strain for which the MIC is ≥2 μg/ml is considered highly resistant (www.srga.org; The Swedish Reference Group for Antibiotics), and many β-lactam agents can reach concentrations considerably higher than these thresholds. Therefore, it is possible for penicillin and other β-lactams to inhibit strains classified as resistant.

Pneumococcal infections due to strains with indeterminate resistance to β-lactam have not been associated with either treatment failure or increased mortality (Heffelfinger *et al.* 2000). Current levels of resistance to penicillin and cephalosporin appear to have little, if any, clinical significance in non-meningeal infections. For treatment of pneumonia caused by indeterminate resistant strains of pneumococcus (0.06≤ MIC <2; www.srga.org), penicillin and its derivatives in standard doses are still effective. In the absence of immediate hypersensitivity reactions, penicillin can be safely administered in doses high enough to overcome these intermediate resistance mutations. For example, treatment failures of β-lactam therapy in pneumococcal pneumonia have never been reported for penicillin, and only rarely reported for other agents that are less active against pneumococci (Buckingham *et al.* 1998; Daum *et al.* 1994; Dowell *et al.* 1999; Fuller *et al.* 2005; Pallares *et al.* 1995; Sacho *et al.* 1987). This is in contrast to the numerous reports of clinical failures in patients with pneumococcal meningitis caused by isolates with penicillin MICs ≥0.12 μg/ml. Only when the strain is known to be fully susceptible to penicillin, it can be used for treating pneumococcal meningitis. As antimicrobial penetration into the cerebrospinal fluid (CSF) is constrained by the blood-brain barrier, even strains with indeterminate resistance are associated with clinical failure. Consequently, the penicillin resistance breakpoint for pneumococci in meningeal infections is defined as MIC ≥0.06 (www.srga.org).

For the macrolides, there are several case reports documenting treatment failure against resistant strains, and studies suggesting an association between macrolide resistance and breakthrough bacteremia (Fuller *et al.* 2005). Noteworthy is that it has been observed that macrolide resistance due to mutations in genes encoding rRNA

and/or ribosomal proteins may result in the emergence of resistance during therapy and subsequently clinical failure (Butler *et al.* 2003; Kays *et al.* 2002; Musher *et al.* 2002). Furthermore, therapy failures have been recorded repeatedly for fluoroquinolones, TMP-SMX, and tetracycline for treatment of pneumonia caused by resistant *S. pneumoniae* (Davidson *et al.* 2002; Empey *et al.* 2001; Hansman *et al.* 1967; Klugman *et al.* 1986; Linares *et al.* 1983; Perez-Trallero *et al.* 1990; Perez-Trallero *et al.* 2003; Urban *et al.* 2001).

1.8.6 Pneumococcal vaccines

Today, two vaccine formulations are available to prevent pneumococcal infections. The immune responses induced by these two different pneumococcal vaccine formulations are further discussed in section 1.6.2. The first developed vaccine has been on the market since 1977 and is a polysaccharide vaccine consisting of the 23 most common capsular serotypes causing invasive disease in the developed world. However, its effectiveness is hampered by poor responses in elderly people, immunocompromised patients, and in very young children (<2 years) as this widely used vaccine only induces T-cell-independent B-cell responses. Therefore, conjugate vaccines have been developed where a carrier protein called CRM_{197}, a non-toxic recombinant variant of diphtheria toxin, is coupled to the polysaccharides which results in a T-cell-dependent response causing B-cells to produce antibodies recognizing the polysaccharide antigens. Pneumococcal conjugate vaccines (PCV) have now been in use for almost 10 years, and several types of PCVs have been developed. The heptavalent conjugate vaccine (PCV-7) targets the serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. These serotypes are together responsible for between 60-90% of pediatric infections in developed countries (Eskola *et al.* 2001). Both the compositions of the 23-valent polysaccharide vaccine and the 7-valent conjugate vaccine were based on the serotype prevalence mainly in the United States. This may cause problems with coverage rates of the vaccines in certain settings and countries. In the least-developed countries, outbreaks of serotypes 1 and 5, which not are included in the PCV-7, are important cause of childhood disease (Reinert *et al.* 2010). Europe and the United States show a similar serotype distribution with only minor differences. By including the serotypes 1 and 5, a 9-valent PCV was developed; and by further addition of serotype 7F, a 10-valent conjugate vaccine could be produced. This was followed by an 11-valent PCV which also included serotype 3. Moreover, the latest PCV formula gives protection against 13 serotypes; besides the ones already mentioned it also targets types 6A and

19A. In the United States, PCV-7 has been in use since 2000 and during that time severe pneumococcal disease has been reduced with nearly 80% among children under the age of five years (Kyaw *et al.* 2006). PCV-7 is now being replaced with PCV-13 in the United States. In Sweden, the heptavalent PCV is included in the national immunization program for children since 2009. PCV-7 is estimated to cover 74% of the strains circulating among children younger than two years in Sweden (Hedlund *et al.* 2003). Just recently, PCV-13 was approved also for use in Europe, and it has potential to give over 90% protections against pneumococcal infections (Reinert *et al.* 2010).

A significant attribute of the PCVs has been the degree of herd immunity (i.e., protection of non-vaccinated persons) it produces. In contrast to polysaccharide vaccines, the PCVs also protect against non-invasive pneumococcal infections (e.g., AOM and sinusitis) and against pneumococcal carriage. The frequency of IPD in non-vaccinated siblings and in adult contacts has been reported to decline after PCV implementation (van der Poll *et al.* 2009). Young children constitute the main reservoir for pneumococci and thus elimination of the carrier state in children reduces transmission risks to the rest of people.

A desired effect of a large-scale use of PCV is decreased prevalence of vaccine serotypes mainly associated with antibiotic resistance. Five of the serotypes (6B, 9V, 14, 19F, and 23F) included in PCV-7 are those most often associated with penicillin resistance (Schrag *et al.* 2000). Kyaw *et al*. reported that IPD caused by resistant isolates were reduced after the PCV-7 introduction in the United States (Kyaw *et al.* 2006), which suggests that vaccination may decrease the prevalence of resistant pneumococci. These results are supported by a study in France that reported a reduction in the carriage of PNSP following PCV-7 introduction (Cohen *et al.* 2009). However, a study from Portugal indicates that vaccination must be combined with a decreased use of antibiotics in order to reduce the resistance rates in pneumococci (Frazao *et al.* 2005). In short term, the vaccine may help to control the spread of resistant pneumococci, but over time the rate of nasopharyngeal carriage of non-vaccine types will probably increase due to serotype replacement. Then the selective pressure imposed by antibiotic use must be reduced to counteract high rates of resistant non-vaccine serotypes.

Several studies have been performed before and after vaccine implementation to monitor the effectiveness and the serotype distribution. A study performed in Netherlands two years after introduction of PCV-7 reported a decrease in vaccine-serotype IPD by 90% in children of age eligible for PCV-7, however, simultaneously,

non-vaccine serotype IPD increased by 71% (Rodenburg *et al.* 2010). For other age groups the IPD frequency did not change. Isaacman *et al*. reviewed several studies from studies in Europe from 1990 to 2008 and they observed that effective use of PCV-7 causes a general decline in IPD in young children and also a decreased prevalence of vaccine serotypes (Isaacman *et al.* 2010). In conclusion, several other studies have shown a general decrease in IPD after vaccine implementation, whereas non-vaccine serotypes have increased. Since PCV-7 introduction, the most common IPD isolates in Europe are 1, 19A, 3, 6A, and 7F (Isaacman *et al.* 2010), which are included in the PCV-13 formula. In the United States, the serogroups 15 and 33 have become an increased cause of IPD in children after PCV-7 implementation (Gonzalez *et al.* 2006). However, IPD caused by vaccine serotypes in children were reduced by 75% after vaccine introduction in the United States (Kaplan *et al.* 2004). The same study reports an increased frequency of serogroups 15 and 33, as well as increased penicillin resistance among non-vaccine serotypes.

The shortcomings of the polysaccharide and conjugate vaccines have prompted extensive research aimed at development of vaccines based on pneumococcal proteins that contribute to virulence and are common to all serotypes. Such serotype-independent vaccine should be highly immunogenic and elicit an immunological memory in young children, who respond well to T-cell-dependent protein antigens. Besides, an optimal antigen should have a low antigenic variability. Several candidate protein antigens have been examined for vaccine potential. For example pneumococcal surface protein A, pneumolysin, pneumococcal surface protein C (also called CbpA), pneumococcal surface antigen (Tai 2006) and pneumococcal neuraminidase (Simell *et al.* 2006) have been proposed as vaccine antigens. To achieve the advantages of herd immunity, an optimal vaccine formula also needs to protect not only against pneumococcal disease but also carriage.

1.8.7 Influenza vaccination

Influenza vaccination is an important measure to reduce the risk for pneumonia, especially in elderly. Likewise, the incidence for pneumococcal pneumonia appears to be decreased by influenza vaccination. A Swedish study reported that influenza vaccination gives a 58% protective efficacy against IPD among elderly; however, the number of patients included was too small to achieve a statistical significance (Christenson *et al.* 2004).

1.9 THEORETICAL BIOLOGY

Applying mathematics in natural sciences and other disciplines have a long history. Just recently there has been an explosion of interest in the use and application of theoretical approaches such as mathematics and computation within biology and medicine. There are numerous reasons for this, for example the explosion of data-rich information sets due to the genomics revolution, which are difficult to understand without the use of analytical tools, and an increase in computing power which enables calculations and simulations to be performed that were not previously possible. Besides, there is an increased interest in *in silico* experimentation due to ethical considerations, risks, unreliability, and other complications involved in human and animal research.

A novel branch in this research area is systems biology, which can be described as a biology-based interdisciplinary study field focusing on complex interactions in biological systems. It applies a new holistic perspective aiming at putting together rather than taking apart. Systems biology encompasses a wide range of fields from modeling of biological system to biostatistics and computational genomic. Systems biologists investigate the behavior and relationships in a certain biological system while it is functioning, and want to illuminate biological processes at the level of systems.

1.9.1 Mathematical modeling of biological systems

Modeling biological systems is a highly relevant task within systems biology. Models can be used to make predictions and to conduct experiments *in silico*. Moreover, the *in silico* design may reduce the time and costs needed to develop medical drugs and also the number of animal experiments. A model is a tool which helps better understand the biological phenomenon studied.

Any model can be defined as a simplified representation of certain aspects of a real system. A model constructed using mathematical concepts, such as equations and functions, is referred to as a mathematical model. Creating mathematical models means moving from the real world into an abstract world of mathematical concepts which is where the model is created. The model is then manipulated, solved and analyzed in several ways using mathematical, computational, and statistical techniques. At last, the real world can be re-entered with the solution to the mathematical problem, which is then translated into a useful explanation to the real problem. For a particular problem there is not only one right and proper model; many different models can be developed for dealing with the same problem. However, some models may be better than other in

the sense that they are more useful or more accurate. In general, the success of a model depends on how easily it can be used and how accurate its prediction is. Any model will also have a limited range of validity and should not be applied outside its range. A mathematical or computational model of a biological system is developed based on data and knowledge of the function and relationships within the system of interest (Figure 7).

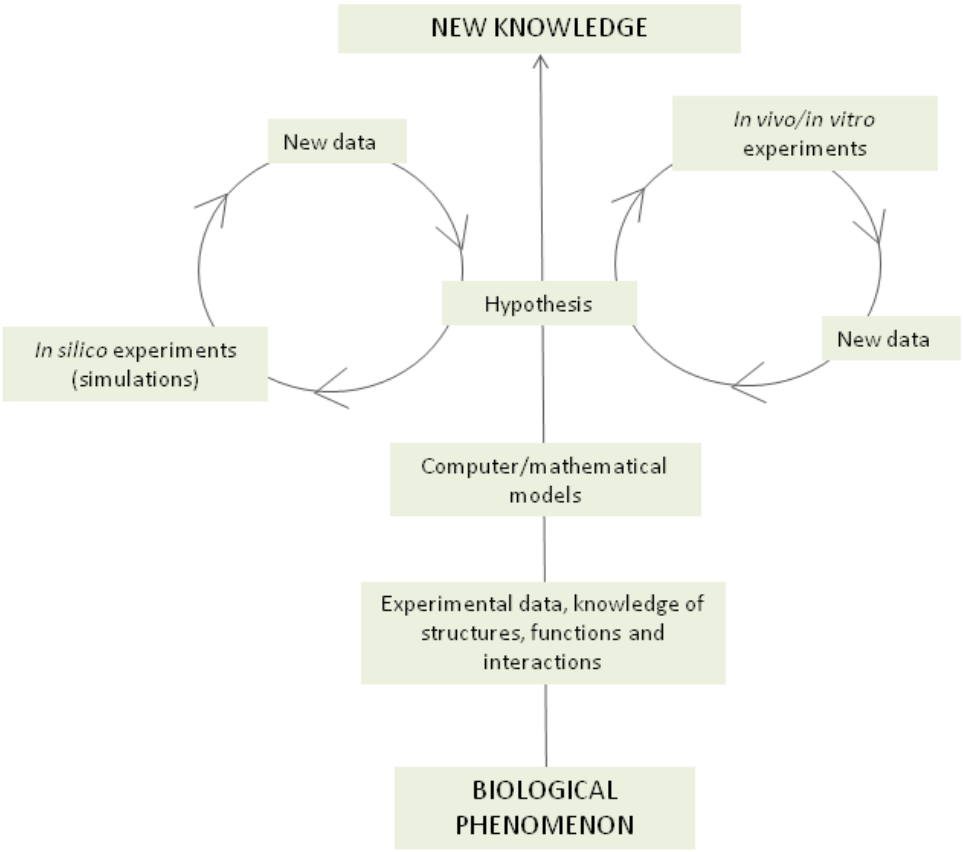

FIGURE 7 The process of knowledge generation in systems biology. Adapted from Reiss, R., Systems of Life. Systems Biology. (2002) Druckerei Hörning, Heidelberg.

The model permits hypotheses about the characteristics and behavior of the system to be derived. These hypotheses can then be verified in parallel in *in silico* and *in vivo/in vitro* experiments. The *in silico* simulations will generate new data that allow validation of the hypotheses. In parallel, *in vitro* experiments will produce new biological data which will also help to verify the hypotheses. This iterative work process leads to new biological knowledge.

1.9.2 Modeling approaches

There are several different modeling approaches suitable for different applications. These approaches can be categorized broadly as being deterministic or stochastic. A deterministic model is one in which each variable changes according to a mathematical formula, without any room for random variation. In such models, a given input will always generate the same outcome. Examples of deterministic approaches include difference equations (discrete time, continuous state space), ordinary differential equations (ODE; continuous time and state space, no spatial derivatives), and partial differential equations (continuous time and state space, spatial derivatives).

The deterministic modeling approach contrasts with the stochastic (probabilistic) one where randomness is present and variables states are described using ranges of values in the form of probability distributions. Approaches that encompass stochastic processes are Markov models and Bayesian networks.

1.9.3 Models of infectious disease epidemiology

For a long time, mathematics has been an important tool in infectious disease epidemiology. Models within infectious disease epidemiology can be used for predictions about the future, but also aid us in understanding complex networks by providing a simplified picture of a situation or a progression. They may help us to recognize which determinants are the most important factors of the progression and therefore which determinants we should examine more in detail. Infectious disease models permit the conductance of *in silico* experiments that would be practically feasible or unethical to perform in a controlled experiment in real life. For example, we cannot intentionally introduce pathogen agents into populations or withhold potentially lifesaving interventions for the sake of scientific study. The use of mathematical models of disease transmission provides a way around this difficulty, and thereby makes it possible to systematically evaluate intervention strategies such as vaccination and quarantine.

The progress of an epidemic in a large population has traditionally been modeled using compartmental models. In such models, the population diversity is reduced to a few key characteristics which are relevant for the infection under consideration. As example, we can use childhood diseases, such as measles, mumps, and rubella, which usually provide long-lasting immunity after infection. The population can then be subdivided into those who are susceptible to the disease, those who are currently infected or infectious, and those who have recovered and are immune

(Nichol *et al.* 2010). Such subdivisions of the population are called compartments. Standard convention labels these three compartments S (susceptible), I (infected), and R (recovered), and therefore this model is referred as the SIR model. Each individual of the population typically progresses from susceptible to infectious to recovered as shown in Figure 8. Between susceptible and infected, the transition rate is β. This rate considers the probability of acquiring the disease in a contact between a susceptible and an infected individual (Nichol *et al.* 2010). The transition rate from infected to recovered is denoted v, the recovery rate. If an infected person becomes immune after time D, the duration of the disease, then $v = 1/D$. For example, if the disease duration, D, is 7 days, then 1/7 of all the infected individuals recover each day and move from the infectious compartment into the recovered group.

FIGURE 8 Diagram of the SIR model. The modeled population is subdivided into three compartments as represented by the boxes whereas the arrows indicate transition between the compartments.

However, some of the assumptions underlying the SIR model are seriously unrealistic. For example, it assumes that every people in the population will meet every other with equal probability. This "law of mass action" is actually derived from chemistry and describes how substances enter into chemical reactions. Among individuals there will always be some contacts that are likely or common, and others that will never occur. The contact pattern in the society is far from homogenous and therefore, most infections spread more slowly than predicted by the SIR model (Giesecke 2002). Another unrealistic assumption is that only one type of contact exists as given by β, although diseases may spread along different routes with varying probabilities of transmission to occur.

Such a deterministic modeling approach implies that the progression and size of an epidemic are always determined by the defined transition rates, and will generate the same result every time. For most epidemics, however, it is intuitively clear that chance may play a role. Probabilistic models are often more complex than deterministic ones, and is generally evaluated using a computer. A simulated epidemic is then run repeated

times. Each run produce different results due to chance and these results can be averaged to find the most likely outcome.

One type of a probabilistic approach is contact network modeling which has the advantage that it captures realistic diversity in contact patterns which are ignored by most compartmental models. A realistic network model of the contact patterns is then created and the spread of an infectious agent can be predicted based on intrinsic features of the pathogen and structural properties of the network. Nonetheless, the main problem for both deterministic and probabilistic models is the lack of good quantitative data of infections (Giesecke 2002). Besides, the contact pattern in the population is sometimes unknown and very complicated to model.

2 AIMS

The general aim of this thesis was to learn more about emergence and spread of antibiotic resistant pneumococci using both theoretical and empirical methods.

2.1 PAPER I

The increasing prevalence of antibiotic resistant pneumococci is primarily due to the spread of resistant strains belonging to few clones. Thus, the aim of **paper I** was to evaluate measures aiming to control transmission of *S. pneumoniae* in the community.

2.2 PAPER II

The effects of reduced penicillin consumption on the pneumococcal population dynamic are not fully understood. In **paper II,** the impacts of penicillin consumption and between-strain competition on the spreading of susceptible and non-susceptible pneumococcal strains in the community were assessed.

2.3 PAPER III

Competence for genetic transformation is an important mechanism for spread of resistance genes in pneumococci. Understanding the regulation of competence is essential to prevent horizontal spread of resistance genes. **Paper III** aimed at studying the dynamic and regulation of competence in pneumococci.

2.4 PAPER IV

Invasive infections caused by antibiotic resistant pneumococci constitute a worldwide problem. The aim of **paper IV** was to examine the resistance pattern in invasive clinical isolates of *S. pneumoniae* collected in southwest Sweden during 1998-2001, and correlate resistance to clinical parameters.

3 MATERIAL AND METHODS

3.1 COLLECTION OF STRAINS

During 1998-2001, pneumococcal isolates from blood and CSF were collected prospectively from the six laboratories of clinical bacteriology in the counties of Västra Götaland and Halland in south-west Sweden. All 13 hospitals in the counties were served by these laboratories. In total, 839 clinical strains were isolated from patients suffering from invasive pneumococcal infection, and 827 strains could be revived for susceptibility testing.

3.2 CLINICAL DATA

In **paper IV**, including 827 invasive pneumococcal isolates, clinical data were available. Information about age, gender, mortality, underlying disease, known abuse of alcohol and/or narcotics, clinical manifestation, year of isolation, geographic area were recorded. A more detailed description of the clinical characteristics and serotype distribution on the same material has been published in a previous report (Berg *et al.* 2006). Complete records of clinical data were obtained from individual hospital notes for 818 patients. For the remaining nine patients, date of culture, source of isolate, age and gender were known.

3.3 METHODS

3.3.1 Serotyping

All pneumococcal isolates were serotyped with the quellung reaction at the WHO Collaborating Centre for Reference and Research on Pneumococci, Statens Serum Institute, Copenhagen, Denmark (Lund 1978).

3.3.2 Antibiotic susceptibility

The pneumococcal isolates were tested for antimicrobial susceptibility using the epsilometertest (E-test®) to determine MIC to penicillin G, erythromycin, clindamycin, tetracycline, moxifloxacin, and TMP-SMX. The E-test® (AB Biodisk, Sweden) is an antibiotic gradient strip that is applied to an inoculated agar plate and results in an elliptical zone of inhibition that intercepts the graded strip, generating a quantitative

result. The gradient ranges for the antimicrobial agents included in this study were as follows: penicillin G 0.002-32 μg/ml; erythromycin 0.016-256 μg/ml; clindamycin 0.016-256 μg/ml; tetracycline 0.016-256 μg/ml; moxifloxacin 0.002-32 μg/ml; and TMP-SMX 0.002/0.038-32/608 μg/ml. The isolates were categorized as sensitive (S), indeterminate (I) or resistant (R) for all agents according to the SRGA system of species related breakpoints (www.srga.org).

3.3.3 Methodological considerations

E-test® appears to be a reliable method for determination of MICs for a variety of antibiotics among pneumococci (Jacobs *et al.* 1992). However, there are methodological problems associated with this test for estimating the MICs of pneumococci to cotrimoxazole (Jorgensen *et al.* 1991) since poor interlaboratory reproducibility have been reported (Fuchs *et al.* 1997; Lovgren *et al.* 1999).

3.4 THEORETICAL METHODOLOGY

All simulation procedures in **paper I** and **II** were programmed in MATLAB (Mathworks Inc.), whereas SPSS (SPSS Inc.) were used for the statistical analyses. In **paper III**, the calculations were performed with MATLAB and Mathematica (Wolfram Research Inc.).

3.4.1 Contact network epidemiology

In this section, the methodology underlying contact network modeling is discussed briefly. The method of contact network epidemiology used in **paper I** and **II** can be divided into three steps. First, a realistic network model of the contact patterns at an appropriate scale is created. Next, the infection is spread computationally through the population based on intrinsic characteristics of the microbe and structural properties of the network. Thereafter, the network is manipulated to model control strategies. The epidemiological consequences of such manipulations can then be analyzed. Figure 9 graphically visualizes the network concept.

The patterns of interactions that may lead to transmission of an infectious disease are captured by a contact network model. Herein, each individual translates into a vertex and contacts among people into edges that connect appropriate vertices. For example, one may like to model the contacts between persons in a hospital that may result in respiratory disease transmission or the sexual interactions within a high school that

might lead to sexually transmitted disease transmission. When simulating the spread of infectious agents in network models, an infection may first appear either at randomly chosen vertices or at predetermined vertices. Disease will propagate with certain transmission probabilities through the network as defined by the edges. The vertices will remain infected and infectious for some period of time, during which they have the potential to transmit disease to each of their contacts. Both the level of contagion and the structure of the contact network influence the percolation of disease through a network. The disease will traverse some but not all of the edges.

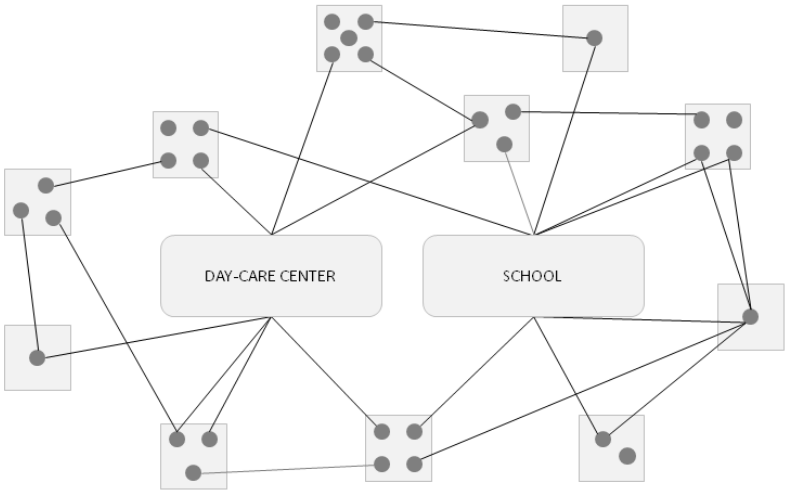

FIGURE 9 Graphic illustration of the network concept. The dots represent individuals in the population, and the quadratic boxes are households whereas the rectangles are DCC and school, respectively. Individuals within same household have contact with each other, while individuals within same DCC or school only have contact if they belong to the same day-care group or class. Disease transmission can only occur via edges, i.e., contacts, between vertices, i.e., individuals. The edges are bidirectional, i.e., disease may be transmitted in both directions.

By manipulating the contact network model, we can evaluate the impact of control strategies. There are several forms of interventions. One is transmission-reducing action and includes interventions that in one way or another decrease the probability of transmission (e.g., improved hand hygiene, disinfection, face mask etc.). Another way is immunizing interventions such as prophylactic medication and vaccination. More specifically, different vaccine strategies (e.g., vaccinating specific groups of individuals based on risk factors such as age or health, general vaccination) can be assessed by simply removing the immunized individuals from the network, whereas isolation of infected persons belongs to contact-reducing interventions and can be modeled by removing appropriate edges between vertices.

3.4.2 Systems of ordinary differential equations

An ODE is a mathematical equation for an unknown function x of one variable that relates the values of the function itself and its derivatives of various orders. In **paper III,** a dynamical system of non-linear ODEs describing the components involved in the competence-evoking quorum sensing pathway was constructed. This model was derived in a biology-driven way, which means that the underlying biology was studied and then translated into reactions and equations.

The main components in ODE models are the objects and the actions. The objects may represent model entities such as molecules, cells or organs, while the actions may represent activation, inhibition, or migration. ODEs describe the rates of changes (actions) of quantities (objects). In the competence model, the variables denoted the total amounts of the components in a population of n cells, whereas the parameters represented rates of changes.

3.4.3 Parameter analysis

Parameter analysis can be used to examine how sensitive a model is to changes in the value of the parameters or the structures of the model. Usually, parameter analysis is performed by a series of tests in which different parameter values are set in order to observe how a change in the parameter causes a change in the model outcome. Parameter analysis is a technique for systematically changing parameters to determine the effects of such changes. Dependent on the current model type, there are numerous procedures of how to change the parameter values and also of how the results are to be analyzed.

4 RESULTS AND DISCUSSION

This thesis aimed at investigating emergence and spread of antibiotic resistant *S. pneumoniae* using both theoretical and empirical methods. In **paper I,** an individual-based network model was developed and used to evaluate the efficacy of interventions aiming to control pneumococcal transmission in the community. A similar modeling approach was applied in **paper II** wherein the impact of penicillin consumption on pneumococcal spreading in the society was investigated. Although clonal spreading serves as the primary mechanism responsible for the emergence of resistance in pneumococci, the pneumococcal feature of competence for genetic transformation is also of importance. In **paper III**, the regulation of the competence-evoking quorum sensing network in pneumococci was studied using theoretical means. Finally, in **paper IV**, the resistance pattern among invasive clinical isolates collected prospectively and correlations between resistance and clinical parameters was investigated.

4.1 PAPER I

In **paper I**, an individual-based network model was constructed by employing demographic data from Sweden during the mid-2000s. An *in silico* population consisting of 25,000 individuals were set up and each individual was assigned age according existing data. Contact sites included were households, DCCs, school classes, and other contacts, which implicates close contacts occurring that may transmit pneumococci, for example interactions between children and their grandparents. The model considered asymptomatic carriers as the transmission reservoir for pneumococci in the community. Individuals colonized with pneumococci can transmit this microbe to susceptible individuals with whom they have an existing contact as defined by the contact network. Transmission occurs between individuals with certain probability depending on the contact type and age of the exposed individual. Duration of carriage appears to vary widely, however, an inverse relationship between age and carriage duration has been observed (Ekdahl *et al.* 1997). Each colonized individual was assigned a carriage duration at the time point for colonization, where the duration was drawn from an exponential distribution with a mean dependent on the age of the individual. The model was validated by comparing the age distribution for prevalence

of pneumococcal carriage obtained from computer simulations with observed data (Hogberg *et al.* 2007) (Figure 10).

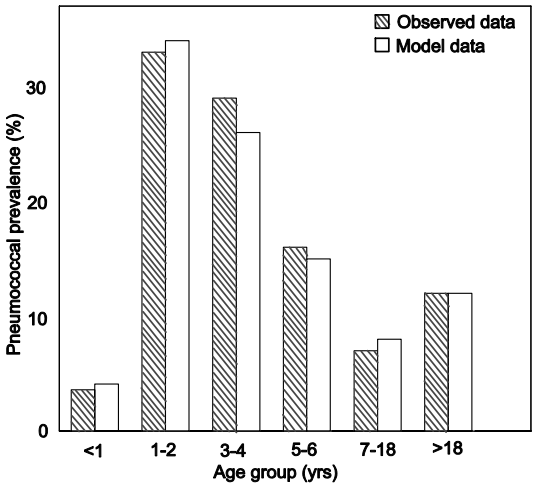

FIGURE 10 Age distribution among pneumococcal carriers. The carriage prevalence in different age groups obtained from model simulations were compared with observed data (Hogberg *et al.* 2007).

To elucidate which of the model parameters affects the transmission process mostly, a parameter analysis was performed. For each parameter tested, one hundred simulations were performed with varying values of the parameter of interest, while keeping all others parameter unchanged. The parameter values were given by randomly assigning from a normal distribution with a mean equal to its default value and a standard deviation of 10% of the parameters default value. By calculating elasticity coefficients for individual parameters, we were able to assess their impact on pneumococcal transmission. Three parameters were revealed as key determinants: *Carriage duration*, *Day-care transmission probability,* and *Group size in the DCCs*. Duration of carriage has been reported to vary with pneumococcal serotype besides host factors. Although carriage duration is an important factor, it is difficult to affect and therefore cannot be considered as an interventional option. Also transmission probability in the DCCs appears to be highly relevant and may probably be lowered by improved hygiene routines. Further studies are required to identify more specific factors affecting the DCC transmission probability. The sizes of DCC groups seem to possess a very important role in pneumococcal transmission dynamic. Reduced DCC group sizes on community-level in the model, resulted in a distinct decline in pneumococcal transmission. This concurs with a study reporting that the pneumococcal carriage rate is much higher in DCC facilities attended by more than or equal to 45 children, than in centers with less than 45 children (Rosen *et al.* 1984). We also simulated three different

intervention scenarios: 1) Varying proportion of children attending day-care; 2) Different sizes of day-care groups; and 3) Restriction of day-care attendance for pre-school children. The scenario simulations support the importance of the group size in DCCs. A decrease from 16.7 to 13.4 children at average for the DCC group size, the model predicts a reduction in transmission of 82%.

4.2 PAPER II

In **paper II** the spreading dynamic for pneumococci was further studied. The starting point was an individual-based network model similar that constructed in **paper I**. The *in silico* population was extended to comprise of 50,000 individuals, and the model considered spread of two competing pneumococcal clones; one clone was assumed to be fully susceptible whereas the other one was defined as PNSP. Outpatient penicillin users were initiated according available data of penicillin sales in Sweden during 2008 for different age groups. If an individual who received penicillin at the same time was carrying pneumococci, different outcomes were possible dependent on the resistance characteristics of the colonizing clone. Carriages of susceptible strains were considered to be cleared in 80% of the cases, while PNSP carriages were assumed not to be eradicated by penicillin treatment. The model also considered the progression from carriage to clinical disease requiring outpatient penicillin treatment. The calculated risks for disease progression were only based on the age and did not considered any other risk factors. The risks for disease progression were estimated by determining the ratio between the age-specific incidence of pneumococcal disease and the incidence of pneumococcal carriage in defined age-groups. In Figure 11, the estimated disease risks for different age groups are presented.

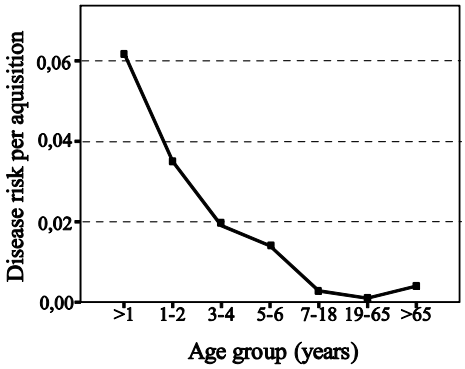

FIGURE 11 Age-related risks for disease progression per individual colonization event.

Simulations of the model revealed that the age distribution for PNSP carriage prevalence is affected by penicillin consumption, whereas the age distribution of carriage prevalence appears not to be changed for the susceptible strain. The proportional prevalence increased for children between one to two years of age while it decreased for adults >18 years (Figure 12).

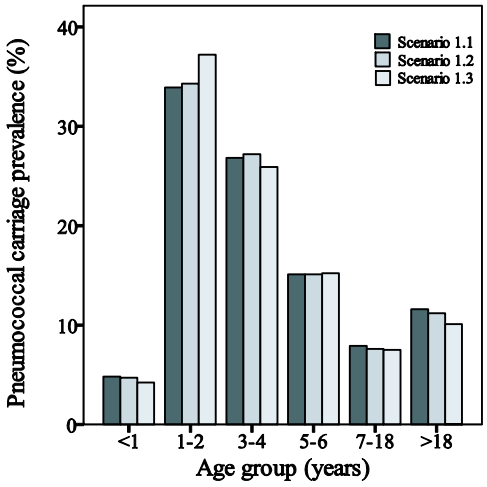

FIGURE 12 Age distribution for pneumococcal carriage prevalence for different model scenarios. Scenario 1.1 reflects spread of a susceptible strain in non-penicillin consuming population; scenario 1.2 represents spread of a susceptible strain under the selection pressure of penicillin; and scenario 1.3 shows spread of a PNSP strain under the selection pressure of penicillin.

The absolute incidence of PNSP, however, increased in all age groups under selective pressure of penicillin. Furthermore, simulations also showed that decreased penicillin usage in pre-school children (age 0-4 years) had an almost as extensive impact as reducing the overall penicillin consumption. This suggests that this age group should be the primary target for reducing unnecessary penicillin use in order to decrease the selection pressure on PNSP.

According to the model, it appears very difficult to reduce the absolute frequency of PNSP simply by controlling the penicillin consumption assumed that penicillin resistance does not confer any fitness cost. For resistant organisms lacking such a biological cost, transmission control measures are very important. Examples of interventions aiming to reduce pneumococcal transmission include hygienic measures, vaccine programs, identification and isolation of individuals carrying PNSP, and reduced group size in DCCs (**paper I**). Introduction of national immunization programs will decrease the vaccine serotypes mainly associated with penicillin resistance; five of

the seven serotypes included in the 7-valent vaccine (6B, 9V, 14, 19F, and 23F) are those most often associated with penicillin resistance (Schrag *et al.* 2000). Combining large-scale use of vaccines with a more restrictive penicillin policy decreases the risk of selecting resistant non-vaccine types.

4.3 PAPER III

In **paper III**, a mathematical model of the regulation of competence was developed. The model consisted of non-linear ODEs describing the components involved in the competence-evoking pathway. Parameter values were assigned from published data as far as possible, however, experimental data could not be found for all. Instead, those parameters were estimated from biological plausibility. All parameters were tested in a wide range of values proving that the qualitative behavior of a system is determined chiefly by the network structure rather than specific parameter values. In the model, the quorum sensing regulated dynamic were investigated by increasing the extracellular concentration of CSP in the model which in turn affected the level of phosphorylated response regulator in the bacteria (Figure 13).

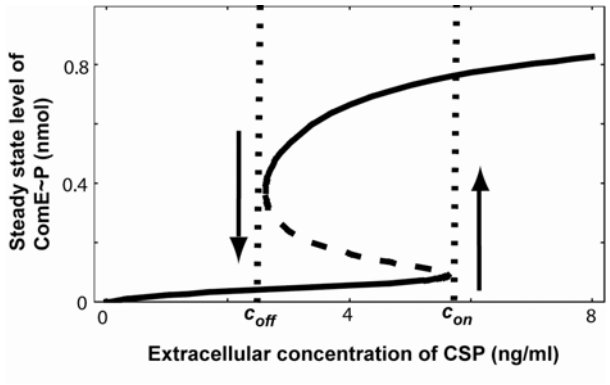

FIGURE 13 Steady state amount of ComE~P as a function of extracellular concentration of CSP. At low CSP concentration, ComE~P has a low steady state level and the *com* system is not activated. As CSP reached a certain threshold value (c_{on}), the steady state level of ComE~P jumps up to a higher value and will only go back to the lower steady state if CSP concentration decreases enough to pass another switch point (c_{off}).

At low CSP concentration, the steady state level of ComE~P is low. As CSP reaches a certain threshold concentration (c_{on} in Figure 13), the level of ComE~P increases and the *com* system becomes activated. The system is only turned off if the CSP concentration decreases to levels much lower than the concentration required for activating the system (c_{off} in Figure 13). Changing the control parameter *CSP* may

switch the system state between the two stable branches. Such discontinuous toggle switches are called hysteresis, which may also be described as the lag between making a change and the response or the effect of that change.

We sought to point out mechanisms responsible for the shut-down of competence, and therefore an elasticity analysis was performed to examine the impact of the individual parameters. The elasticity was investigated in the fold bifurcations (c_{on} and c_{off} in Figure 13) of the hysteresis curve by computing the elasticity of each parameter. From this analysis, the off-switching fold appears to be most sensitive to changes occurring at the transcriptional level suggesting that the down-regulation of competence probably is mediated by a repressor acting on the *comCDE* operon. Next, the model was extended by introducing a repressor shutoff mechanism. Oscillating dynamics in the pneumococcal competence was then observed. Depending on the parameter values, the system was demonstrated to exhibit damped or sustained oscillations (Figure 14). This dynamic is in consistency with previous experimental studies which have reported waves of competence in a pneumococcal batch culture (Chen *et al.* 1987; Morrison 1997).

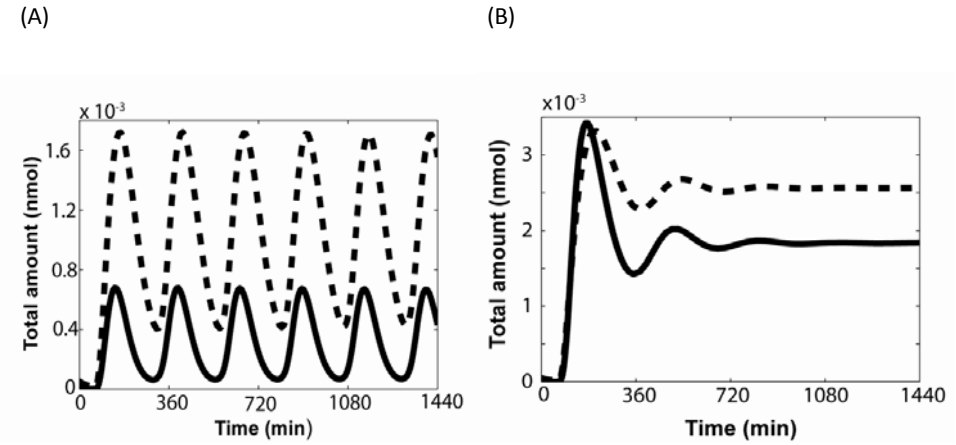

FIGURE 14 Sustained (A) and damped (B) oscillations in the competence system. By introducing a repressor in the model, competence appeared in waves. The dashed lines indicate the total amount of repressor, while the solid line represents the total amount of ComE~P.

4.4 PAPER IV

During 1998-2001, a total of 839 pneumococcal isolates from blood and CSF were collected prospectively in the counties of Västra Götaland and Halland in south-west Sweden (Berg *et al.* 2006). Of the 839 collected strains, 827 could be revived for

susceptibility testing. In **paper IV**, MIC-values to penicillin G, erythromycin, clindamycin, tetracycline, moxifloxacine, and TMP-SMX were determined using E-test®. Isolates were classified as sensitive (S), indeterminate (I) or resistant (R) for all agents according to the SRGA system of species related breakpoints (www.srga.org). We found that 90% of the strains could be classified as S to all agents, whereas 10% were I or R to at least one antimicrobial agent. Of these 83 strains, 62 were non-susceptible to only one antibiotic and the remaining 21 strains were non-susceptible to more than one agent. 2.7% of the isolates had reduced susceptibility to penicillin; none of these was R. For the other agents, the resistance proportions were as following: clindamycin 0.7%; tetracycline 1.9%; moxifloxacine 0.1%; and TMP-SMX 4%. Reduced susceptibility to at least one agent was compared with age, gender, clinical manifestation, underlying diseases, year of isolation, geographic region, and outcome, but no correlations were found. The serotype distribution differed between susceptible and non-susceptible isolates (Table 1).

TABLE 1 Reduced susceptibility to any of the antibiotics tested among the most prevalent serotypes in *S. pneumoniae*.

		Reduced susceptibility to any agent		
Serotype	**Total no. of strains**	**No.**	**(%)**	***p*-value**
1	118	7	6	ns
7F	88	0	0	0.0001[a]
9V	71	12	17	ns
14	70	28	40	<0.0001[b]
4	68	0	0	0.001[a]
12F	48	3	6	ns
6B	36	6	17	ns
3	34	0	0	0.04[a]
23F	34	4	12	ns
8	30	0	0	ns
9N	29	1	3	ns
6A	28	2	7	ns
19A	26	5	19	ns
22F	24	1	4	ns
19F	21	7	33	0.003[b]
Other serotypes	102	7	7	
All serotypes	827	83	10	

[a] Significantly lower; [b] significantly higher when compared with all other serotypes.

Significantly lower proportions of serotypes 7F, 4, and 3 were I or R to at least one agent when compared with all other serotypes. In contrast, significantly higher

proportions of serotypes 14 and 19F had reduced susceptibility to at least one agent in comparison with all serotypes.

The vaccine coverage rates for the respective vaccines among strains with decreased susceptibility against at least one antimicrobial agent (Table 2) were also examined. Vaccine coverage rates are relative high for the strains with reduced susceptibility to any agent, between 73-96% depending on the vaccine. In contrast, the proportion of vaccine-types among the fully susceptible strains is only 39% for the 7-valent vaccine.

TABLE 2 Vaccine coverage rates for all strains and for the strains with reduced susceptibility, respectively.

Vaccine	All 827 strains Vaccine types /all strains (%)	All 83 strains with reduced susceptibility to ≥1 agent Vaccine types/all non-susceptible strains (%)
7-valent	350/827(42)	61/83 (73)
10-valent	557/827 (67)	68/83 (82)
23-valent	781/827 (94)	80/83 (96)

[a]$p<0.0001$; [b]$p=0.003$.

The results of this study are generally in agreement with previous studies of invasive isolates from Sweden (Ekdahl *et al.* 1998; Hedlund *et al.* 2003; Hedlund *et al.* 1995; Sandgren *et al.* 2004). Probably, the low sales of antimicrobials compared with other countries (Cars *et al.* 2001) have contributed to the relatively favorable resistance situation in Sweden.

5 CONCLUSIONS

- One of the most efficient strategies to reduce pneumococcal carriage and spread in the community seems to be decreasing the group sizes in the DCCs, i.e., fewer numbers of children per class room.

- The penicillin consumption in the community appears to affect the age distribution of carriage prevalence of PNSP whereas the age distribution among carriers of susceptible pneumococci seems unaffected.

- It seems extremely difficult to reduce the absolute level of PNSP by simply controlling the penicillin usage if PNSP does not confer any fitness cost for resistance. A more restrictive consumption of penicillin together with other control measures, e.g., vaccine programs, is then required to manage the penicillin resistance in pneumococci.

- Decreased penicillin usage in pre-school children (age 0-4 years) appears to have almost as extensive impact as reduced overall penicillin consumption. This suggests that this age group should be the primary target for reducing unnecessary penicillin use in order to decrease the selection pressure on PNSP.

- The molecular understanding of competence regulation is important to be able to prevent horizontal spread of resistance genes. Down-regulation of competence is suggested to occur at transcriptional level, possibly by a repressor acting on the *comCDE* operon.

- The impact of antibiotic resistance in invasive pneumococcal infections remains limited in south-west Sweden. No correlations were found between reduced antibiotic susceptibility and age, gender, clinical manifestation, underlying diseases, year of isolation, geographic area or mortality.

- The serotype distribution differs between invasive pneumococci with reduced susceptibility and fully susceptible pneumococcal strains. Isolates having decreased susceptibility to any of the antimicrobial agents tested belonged more frequently to serotypes included in the 7-valent vaccine.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Pneumokocken är en bakterie som orsakar mycket lidande runt om i världen och anses vara ansvarig för 1-2 miljoner dödsfall varje år. Den ger upphov till allvarliga sjukdomar såsom lunginflammation, hjärnhinneinflammation och sepsis. Men pneumokocken kan även orsaka mindre farliga infektioner som öroninflammationer och bihåleinflammationer. Vanligen är det fullt friska personer, framför allt yngre barn, som bär på pneumokocker utan att uppvisa symtom. Bakterierna koloniserar då bakre näsvägg och svalg, men orsakar ingen skada på sin värd. Emellanåt övergår dock bärarstadiet till sjukdom – exakt varför detta sker vet man inte, men förmodligen spelar både värdfaktorer och den specifika pneumokockens egenskaper in. Dessa symtomfria bärare är dock viktiga ur smittspridningssynpunkt då de för pneumokockerna vidare till andra människor, som i sin tur också blir friska bärare eller utvecklar sjukdom.

Traditionellt sett har infektioner med pneumokocker behandlats med olika typer av antibiotika, framför allt penicillin. Men under de senaste decennierna har det blivit allt mer vanligt med resistenta pneumokocker. Resistens innebär att bakterierna har förändrats så att antibiotika inte längre är verksam mot dem. Den främsta orsaken till uppkomst och spridning av resistenta pneumokocker är den omfattande användningen av antibiotika. I stora delar av världen är resistenta pneumokocker ett betydande problem. I Sverige, där vi varit lite mer försiktiga med antibiotikaanvändning, har vi en lägre förekomst av resistens bland pneumokockerna. Det förekommer även multiresistenta pneumokocker, dvs. pneumokocker som är resistenta mot penicillin och minst två andra antibiotika. Infektioner orsakade av resistenta pneumokocker försvårar och fördröjer effektiv behandling – många gånger är adekvat behandling insatt i tid livsavgörande för patienten. Ökad insikt och kunskap kring uppkomst och spridning av resistenta pneumokocker är därför viktigt för framtiden. Målet med denna avhandling var ökad kunskap kring uppkomst och spridning av resistenta pneumokocker genom att använda teoretiska såväl som empiriska metoder.

I den **första artikeln** konstruerades en kontaktnätverksmodell baserad på verkliga demografiska data som reflekterar dagens samhällsstruktur för ett genomsnittligt Sverige. Genom att bygga upp en fiktiv population, där individerna tilldelas unika egenskaper som ålder och sociala relationer, kan sedan smittspridning simuleras i populationen. Modellens olika parametrar analyserades i syfte att se vilken åtgärd i samhället som är mest effektiv ur smittspridningssynpunkt. Det som framgick

framförallt var betydelsen av gruppernas storlek på förskolorna, dvs. antalet barn per avdelning. Genom att simulera en minskning av antalet barn per förskolegrupp från 16.7 till 13.4 så minskade smittspridningen av pneumokocker i samhället med 82 %.

Denna nätverksmodell utökades i den **andra artikeln** genom att omfatta en större population och inkludera även spridning av två pneumokockstammar som konkurrerar mot varandra. Den ena stammen var penicillinresistent medan den andra var fullt känslig. Antibiotikaförbrukning enligt försäljningsdata i Sverige inkluderades också. Målet var att studera hur penicillinanvändningen påverkar spridningsdynamiken hos pneumokockerna. Simuleringsresultaten visade att den relativa andelen resistenta pneumokocker minskar i samhället vid minskad penicillinanvändning, men inte den absoluta förekomsten vilket kan förklaras genom penicillinets inhiberande effekt på de känsliga stammarna. Vidare studerades även hur penicillin påverkar åldersdistributionen hos pneumokockbärarna. Penicillinanvändning verkar leda till ökad förekomst av resistenta stammar hos de yngre barnen, medan åldersdistributionen för bärarna av de fullt känsliga pneumokockerna inte påverkades.

Den **tredje artikeln** i avhandlingen handlar om en egenskap hos pneumokocken som har bidragit till dess resistensuppkomst. Bakterierna är nämligen kompetenta för naturlig transformation vilket innebär att de kan plocka upp DNA som finns i deras omgivande miljö. DNA:t sätts sedan in i deras eget genom och uttrycks. På så sätt kan pneumokockerna plocka upp genetiska fragment som kodar exempelvis för resistens, från andra pneumokocker men även från andra närbesläktade bakterier. Systemet som reglerar kompetensutvecklingen kallas ComABCDE. I **artikel III** konstruerades en matematisk modell som beskriver detta kompetenssystem. Vi kunde visa att systemet uppvisar ett så kallat hysteresis-beteende. En analys av modellens parametrar utfördes för att undersöka var systemet är mest känslig för störningar, m. a. o. var det skulle kosta minst för bakterien stänga av systemet. Analysen pekade på en repressor som möjlig mekanism och en utökad modell med repressor konstruerades. Kompetensen framträdde då i vågor vilket tidigare hade setts i experiment.

I den **fjärde artikeln** studeras resistensmönstret hos kliniska isolat insamlade från patienter med invasiva pneumokockinfektioner. Denna prospektiva studie omfattar 827 bakterieisolat insamlade under åren 1998-2001 i en region Västra Götaland och Halland. Isolaten analyserades med avseende på kapseltyp samt resistensnivå för sex olika antibiotika. Majoriteten av isolaten, 90 %, var fullt känsliga mot alla inkluderade antibiotika, medan 10 % hade minskad känslighet för minst ett antibiotikum. En liten del av isolaten, 2.7 % visade nedsatt känslighet mot penicillin, men ingen av dessa blev

klassad som resistent. Vi fann ingen korrelation mellan nedsatt känslighet mot minst ett antibiotikum och ålder, klinisk manifestation, bakomliggande sjukdomar eller utgång. Däremot såg kapseltypsfördelningen lite annorlunda mellan de fullt känsliga och de som hade nedsatt känslighet. De serotyper som ingår i det 7-valenta pneumokockvaccinet motsvarade 42 % av alla infektioner, medan motsvarande täckningsgrad för infektioner orsakade av pneumokocker med minskad känslighet för antibiotika var 73 %.

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